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To unite or disconnect-
AmotL2 in tubulogenesis and tumor invasion

Thesis for doctoral degree (Ph.D.)

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TO UNITE OR DISCONNECT

AMOTL2 IN TUBULOGENESIS AND TUMOR INVASION

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Populärvetenskaplig sammanfattning

Alla kroppens organ är uppbyggda av mindre enheter; celler, som tillsammans arbetar för att säkerställa organens och de mer omfattande organsystemens funktionalitet. Ett organ kan bestå av flera miljarder celler, som fysiskt och mekanistiskt måste synkroniseras och länkas samman (både med varandra och underliggande vävnad), för att bilda en funktionell enhet. Exakt hur cellerna kopplas samman och hur små förändringar i enskilda cellers form kan, om synkroniserade, ge upphov till enormt viktiga utvecklingar på vävnads nivå, har hittills inte varit helt känt. I arbetena i den här avhandlingen har vi visat att proteinet Angiomotin Like 2 (AmotL2 p100) är nödvändigt för att binda samman celler och möjliggöra synkroniserade form förändringar t.ex. under bildning av kroppens största blodkärl aortan.

Kontakt mellan närliggande celler kan uppstå via specialiserade proteiner i cellernas omgivande membran. Dessa kontaktpunkter är vidare kopplade till-, och länkar samman cellernas cytoskelett, dynamiska men ändå stödjande och stabiliserande nätverk av fibrer inuti cellen. Arbetena i den här avhandlingen visar att AmotL2 p100 möjliggör sammankoppling av de membranbundna kontakt-proteinerna med cytoskelettet, och vidare av granncellers cytoskelett. På så vis förenas närliggande celler till en gemensam enhet. Dynamiken i fibrerna som utgör cytoskelettet, möjliggör att en cell kan känna av och reagera på mekaniska stimuli från omgivande celler. T.ex. kan tryckande- eller dragande krafter från granncellen leda till förändringar i cellens egenskaper såsom cell delning, -form eller rörelseförmåga. AmotL2 p100 möjliggör koordinering av dessa responser och bidrar på så vis till organ-utveckling, t.ex. expansion av aortan till en funktionell tub.

För att ett organ ska fungera, är det dessutom viktigt att cellerna kan orientera sig i förhållande till varandra och omgivningen; en cell måste veta vad som är upp/ner och in/ut. Genom att cellens interna strukturer fördelas asymmetriskt, kan t.ex. en cell i väggen på ett blodkärl veta vilken sida som ska vara riktad in mot det cirkulerande blodet, alternativt gränsa mot omgivande vävnad och en cell i en bröstkörtel kan veta åt vilket håll den ska utsöndra mjölk. Under tumör utveckling tappas ofta den här asymmetrin, vilket kan leda till att desorienterade celler börjar vandra och invadera närliggande vävnad/ blodkärl.

Tumörer är generellt sämre syresatta än frisk vävnad, vilket kan bero på att de blodkärl som bildas inte är särskilt stabila eller effektiva. Förlust av vävnads organisation och cell asymmetri är speciellt vanligt i dåligt syresatta tumörer/ -tumöromården och verkar vara en anledning till att låg syrehalt i en tumör har korrelerats till högre risk för metastas-bildning och sämre prognos.

I arbete IV i den här avhandlingen visar vi på en mekanism, hur specifikt uttryck av en annan form av AmotL2 (p60) vid låga syrehalter, bidrar till spridning av tumör celler genom att orsaka förlust av cell asymmetri och vävnads struktur. Troligtvis verkar AmotL2 p60 tillsammans med alternativa upptäckta- och för närvarande okända processer, för att underlätta spridning av tumör celler till bättre syresatta områden där de kan bilda metastaser.

Slutligen, vi har studerat de vitt skilda effekterna av två versioner av samma protein. AmotL2 p100 styr organiserad tub-bildning under embryonal utvecklingen, medan AmotL2 p60 leder till oorganiserad tumörväxt och ökad spridning av tumör celler.

Abstract

For an epithelial- or endothelial cell sheet or -tissue to form, individual cells must come together, unite and connect. Contacts to adjacent cells and underlying matrix must be initiated, and the junctional proteins mediating the contacts must be further linked to intracellular cytoskeletal networks. By connecting neighboring cells into one unit, contractile actomyosin filaments allow for a closely interlinked, but yet dynamic tissue. Eventhough essential for developmental processes such as organ formation, the exact mechanism of how the cell-cell junctions connect to the contractile actomyosin network has not yet been completely revealed. In the papers of this thesis, we identify the protein Angiomotin Like 2 (AmotL2 p100) as a linker between the contractile radial actin filaments and VE-cadherin at the adherens junctions. Furthermore, AmotL2 p100 enables controlled- and synchronized morphological alterations of individual cells, which can further result in the creation of new tissue level structures, for example through tubulogenesis. We show the radial AmotL2-mediated actin filaments to be crucial for force-generation during morphological transformation and further aortic lumen expansion.

Morphological transformations usually require organization and collaboration of several processes, such as formation/ disassembly of cell-cell and cell-ECM contacts, establishment/ disruption of apical-basal polarity and polymerization/ disassembly of cytoskeletal filaments. Just like proper regulation of those intra- and inter cellular processes and –signals can result in complex structures with diverse morphologies and functionalities, deregulation of the same signals might cause devastating consequences for the function of an organ and the entire organism.

In Paper IV of this thesis, we could identify a shorter-, hypoxia regulated AmotL2 p60 isoform. We show the actions of AmotL2 p60 to cause retainment of apical polarity proteins in cytoplasmic vesicles, hence preventing the establishment of apical-basal polarity. Furthermore, we could show AmotL2 p60 to weaken cell-cell junctions and sensitize cells to growth factor stimuli. The combined actions of AmotL2 p60 cause tumor cell invasion into the surrounding extracellular matrix (ECM).

In conclusion, we here provide data showing the two AmotL2 isoforms to possess entirely distinct functions, uniting cells into a multicellular structure, and disconnecting cells during tumor invasion, respectively.

List of scientific papers

I. ***AmotL2 links VE-cadherin to contractile actin fibres necessary for aortic lumen expansion.***
Sara Hultin, Yujuan Zheng, Mahdi Mojallal, Simona Vertuani, Christian Gentili, Martial Balland, Rachel Milloud, Heinz-Georg Belting, Markus Affolter, Christian S.M. Helker, Ralf H. Adams, Wiebke Herzog, Per Uhlen, Arindam Majumdar* and Lars Holmgren*. Nat Commun. 2014 May 7;5:3743

II. ***The E-cadherin/ AmotL2 complex controls hexagonal packing of epithelial cells.***
Sebastian Hildebrand, **Sara Hultin**, Aravindh Subramani, Mahdi Mojallal, Xiaofang Cao, Tomas Friman, Arindam Majumdar, John Mpindi, Olli Kalloniemi, Staffan Johansson, Yujuan Zheng* and Lars Holmgren*. *Manuscript*.

III. ***AmotL2 integrates polarity and junctional cues to modulate cell shape.***
Sara Hultin, Victoria Ma, Christin Mieth, Yujuan Zheng, Arindam Majumdar and Lars Holmgren. *Manuscript*.

IV. ***AmotL2 disrupts apical-basal polarity and promotes tumour invasion.***
Mahdi Mojallal*, Yujuan Zheng*, **Sara Hultin**, Stephane Audebert, Tanja van Harn, Per Johnson, Claes Lenander, Nicolas Fritz, Christin Mieth, Martin Concoran, Marja Hallström, Johan Hartman, Nathalie Mazure, Thomas Weide, Dan Grander, Jean-Paul Borg, Per Uhlen and Lars Holmgren. Nat Commun. 2014 Aug 1;5:4557.

* These authors contributed equally to this work.

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List of abbreviations

Amot	Angiomotin
AmotL1	Angiomotin Like 1
AmotL2	Angiomotin Like 2
AMP	Adenosine Monophosphate
AMPK	AMP-activated Kinase
AP-1	Activator Protein 1
APC	Adenomatous Polyposis Coli
aPKC	Atypical Protein Kinase C
Arp	Actin related protein
BM	Basement Membrane
c-Fos	FBJ murine osteosarcoma viral oncogene homolog
CAF	Cancer Associated Fibroblast
CAIX	Carbonic Anhydrase IX
CAR	Coxsackievirus and Adenovirus Receptor
Cas	CRIPR associated protein
Cdc42	Cell division cycle 42
Cdh5	Cadherin5 (VE-cadherin)
CIP	Contact Inhibition of Proliferation
COP	Coat Protein
Crb3	Crumbs homolog 3
CRISPR	Clustered Regularly Interspaced Short Palindromix Repeats
DA	Dorsal Aorta
Dlg	Discs large
Dll	Delta-like
DNA	Doxyribonucleic Acid
dsRed	Discosoma sp. Red fluorescent protein
E-cadherin	Epithelial cadherin
EC	Endothelial Cell
ECM	Extracellular Matrix
EGFP	Enhanced Green Fluorescent Protein
EMT	Epithelial-to-Mesenchymal Transition
EphB4	EPH receptor B4
EPLIN	Epithelial Protein Lost In Neoplasm
ER	Endoplasmatic Reticulum
ER	Estrogen Receptor
ERK	Extracellula- signal Regulated Kinase
EYFP	Enhanced Yellow Fluorescent Protein
FA	Focal Adhesion
FGF	Fibroblast Growth Factor
GAP	GTPase Activating Protein
gata1	GATA binding protein 1
GDP	Guanosine Diphosphate
GEF	Guanine nucleotide Exchange Factor
GLUT1	Glucose Transporter 1

GTP	Guanosine Triphosphate
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
hpf	hours post fertilization
HRE	Hypoxia Responsive Element
ICM	Inner Cell Mass
ISV	Intersegmental Vessel
JAM	Junctional Adhesion Molecule
kdrl	kinase instert domain receptor like
LATS	Large Tumor Suppressor kinase
LEC	Lymphatic Endothelial Cell
Lgl	Lethal (2) giant larvae
MAGI	Membrane Associated Guanylate kinase protein
MAPK	Mitogen Activated Protein Kinase
Merlin/ NF2	NeuroFibromatosis 2
MET	Mesenchymal-to-Epithelial Transition
MO	Morpholino
mRFP	monomeric Red Fluorescent Protein
mRNA	messenger RNA
MST	Mammalian Ste20-like Serine/ Threonine kinase
Mupp1	Multiple PDZ domain protein 1
N-cadherin	Neuronal cadherin
OB-cadherin	Osteoblast cadherin
PAC	Artifical cloning vector from bacterial P1-plasmid
Pals	Proteins associated with caenorhabditis elegans Lin7 protein
Par	Partition defective
Patj	PALS1 associated tight junction protein
PCV	Pericardial Vein
PDZ-binding domain	PSD95, Dlg1 and ZO-1 binding domain
PECAM	Platelet Endothelial Cell Adhesion Molecule
Prox1	Prospero homeobox 1
PSD95	Post-synaptic density protein
PTEN	Phosphatase and Tensin homolog
Rab	Ras-related GTP-binding protein
Rac1	Ras-related C3 botulinum toxin substrate 1
Rasip1	Ras-interacting protein 1
RhoA	Ras homolog family member A
Rip	Rat insulin promoter
RNA	Ribonucleic Acid
ROCK	Rho Associated Coiled-coil containing protein Kinase
ROSA26	Locus used for ubiquotous gene expression in mice
Scribble	Scribbled planar cell polarity protein
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNARE	Soluble NSF Attachment protein Receptor
Tag	large T-antigen transgene

TALEN	Transcription Activator-Like Effector Nucleases
TAZ	Transcriptional co-Activator with PDZ-binding motif
TER	Trans-Epithelial Resistance
tnnt2	Troponin Type 2
TRAPP	Trafficking Protein Particle
UAS	Upstream Activation Sequence
VE-cadherin	Vascular Endothelial cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VHL	Von Hippel Lindau protein
vSMC	vascular Smooth Muscle Cell
YAP	Yes Associated Protein
ZO-1	Zonula Adherens 1

Introduction

All the vital functions of the human body (or organism) are controlled by specialized organs, further organized into more complex organ systems. An organ can be compared to a miniature factory, consisting of multiple smaller units (cells) of different origin and morphology, playing distinct roles. Regardless of their diversity, all the cells building up an organ come together and organize in space, as well as synchronize their actions in time, in order to form a structural- and functional unit. The function of an organ is determined by the unique cells contributing to the tissue (parenchyma), in collaboration and tight interaction with surrounding cells (stroma).

The building blocks of our organs

Tissues of the human body can be categorized into connective-, muscle-, nervous- or epithelial tissue. Epithelial cells line body surfaces and –cavities, giving rise to the gastrointestinal tract, kidneys, breast glands, trachea, skin etc.. These cells bring support and structure to the organs, serve as protection against mechanical forces, and in addition separate different chemical compartments by only selectively allowing para-cellular transport of cells or molecules. Nevertheless, the epithelial cells do not simply form a passive protective barrier, but also define the organs by allowing proper secretion, absorption and transport of water, nutrients and hormones. Organs formed by joined and specialized epithelial cells are responsible for some of the most essential functions of the human body, like the oxygen-carbon dioxide exchange in the alveoli of the lungs, milk production in the breast glands of a mother, and absorption of water and nutrients in the intestine (Guillot and Lecuit 2013). The diversity of epithelial functions are somewhat reflected by different cellular morphology. Epithelial cells are categorized into simple- and stratified epithelia respectively, and further subdivided according to shape into squamous, cuboidal and columnar (Gibson and Gibson 2009). As already mentioned, the parenchyma cannot be solely responsible for the functionality of an organ, instead tight contact with the stroma, which can consist of blood vessels, cells of the nervous system, and supportive connective tissue, is most essential. The vascular plexus transport oxygen and nutrients via the blood, to all peripheral organs of the body. The endothelial cells lining the blood vessels are in many ways similar to simple squamous epithelial cells, allowing for diffusion of oxygen and carbon dioxide over the vessel wall (Lammert and Axnick 2012).

Organization into a larger unit

An initial step during organogenesis, is the organization of cells into multicellular structures. Such organization requires high level of intercellular coordination, involving synchronization of external signals (growth factors etc.), local cell-cell signaling, cell polarization and mechanical signals from both neighboring cells and the surrounding extra cellular matrix (ECM). The multicellular sheet must secure the barrier function, while at the same time preserve the dynamics crucial for tissue homeostasis and controlled organ growth. To achieve desired tissue plasticity, a constant and active control of the above mentioned cellular properties is essential (Macara, Guyer et al. 2014).

Consequences of loss of tissue structure

Even seemingly small alterations of one or several of the mechanisms or signals regulating and controlling organ growth, might lead to devastating tissue level outcomes. The ultimate consequence of anarchic cell growth is cancer, where the tissue has lost its normal control of cell growth- and death equilibrium, and further the ability to keep cells of the tissue united and in tight connection. Sustained cell division and immortalization usually initially give rise to a hyperplastic tissue/ benign tumor, which can as a result of further modifications progress into a more aggressive (malignant) tumor; cancer. Such alterations involve for example; stimulation of angiogenesis and adaptation of invasive- and migratory features (Hanahan and Weinberg 2000).

Cancer has traditionally been considered to be one disease, but actually there are more than 100 different types of cancer. Even within one cancer subtype, the features of individual tumors might differ heavily. Most types of cancer stem from epithelial cells (carcinomas), but cancers can also originate from non-epithelial tissues as in the case of sarcomas (from mesenchymal cells of connective/ supporting tissue), leukemia/ lymphoma/ myeloma (from hematopoietic cells and cells of the immune system), and neuroectodermal tumors (from cells of the central- or peripheral nervous systems) (Ferlay, Soerjomataram et al. 2014). In addition to differences reflecting the origin of the tumor, many tumors show regional differences, with certain areas being more differentiated, proliferative, vascularized, immunogenic or invasive, respectively. Furthermore, the neoplastic cells are in close contact- and constant communication with the cells of the surrounding stroma, making the tumor in many ways similar to an organ consisting of a heterogeneous mixture of cell types (Pickup, Mouw et al. 2014).

The mechanisms found to be of most importance for multicellular tissue development, is also among the ones frequently lost or altered in cancer. This thesis deals with the rise and fall of tissue structure and -organization, and the role of Angiomotin Like 2 (AmotL2) in the mechanisms controlling those events. Special focus is put up on the mechanisms regulating unity of single cells into multicellular sheets and –tissues, how the single building blocks are bridged and connected and further what happens when groups of cells break out of the connective unit into the surrounding tissue.

Mechanisms of multicellular sheet formation

Cell-cell junction formation and cell polarity have been described as the main intracellular properties regulating epithelial- and endothelial sheet formation, and are further features commonly lost throughout tumor progression (Gibson and Gibson 2009) (Kang and Pantel 2013). Cell-cell and cell-ECM junction formation, as well as cell polarity are all events highly dependent upon tight connections to- and organization of the cytoskeleton; dynamic cytoplasmic networks of different types of fibers (Gibson and Gibson 2009). In the following paragraphs the different types of junctions, the cytoskeletal subtypes, as well as the mechanisms underlying establishment of cell polarity will be discussed in more detail.

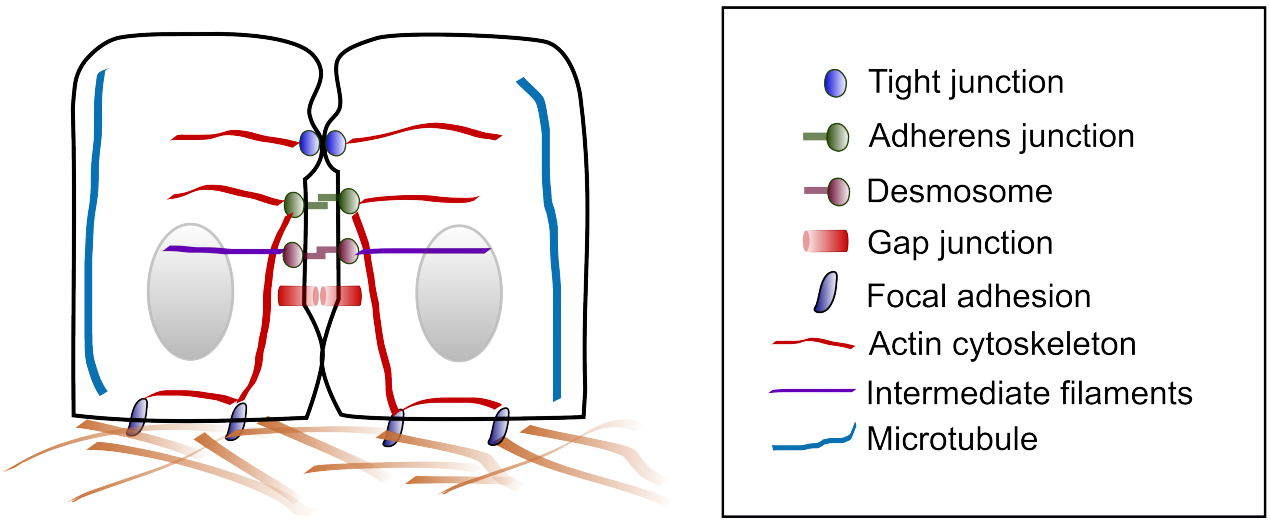

Figure1. Epithelial sheet formation through establishment of cell junctions and apical-basal polarity. Formation of cell-cell- and cell-ECM junctions, apical-basal polarity and organization of the different cytoskeletal subtypes, are coordinated to enable development of a multicellular sheet.

Cell-cell junction formation

The barrier function of a multicellular sheet is highly relying on tight intercellular connections (tight junctions). In addition, cell-cell junctions facilitate intercellular communication, by linking neighboring cells both mechanically through the cytoskeleton (adherens junctions, desmosomes and tight junctions), as well as chemically and electrically through intercellular channels (gap junctions) (Wei and Huang 2013).

Adherens junctions are the first contacts to form in between two adjacent cells. The contacts are initiated by cytoplasmic projections (filopodia) expressing trans-membrane cadherins. As filopodia of the adjacent cells get in close contact, the cadherin proteins expressed at the opposing cell membranes can interact and connect. The initial focal cell-cell contact points are further extended and sealed, to finally form an adherens junction belt. Both the filopodia formation and sealing of the two cell membranes are highly dependent upon cadherins being connected to the actin cytoskeleton. In a newly formed and immature junction cadherins associate solely to contractile radial actin filaments. However, as the junction matures it becomes further stabilized by an apical circumferential actin belt (Abe and Takeichi 2008).

The superfamily of transmembrane cadherin proteins is, as the name implies, a large protein family consisting of classical cadherins, protocadherins and cadherin-related proteins. The classical cadherins is by large the most studied and comprises Epithelial (E)-cadherin, Neuronal (N)-cadherin, Placental (P)-cadherin, Retinal (R)-cadherin, Muscle (M)-cadherin, Osteoblast (OB)-cadherin and Vascular Endothelial (VE)-cadherin (van Roy 2014).

Desmosomes are present in epithelial-, but not in endothelial cells, and are usually initiated at sites bordering to the newly formed adherens junctions. These junctions are constructed out of specialized cadherins, and are more robust and hence less dynamic compared to the adherens junctions. The enhanced stability of the desmosomes is due to their association to intermediate filaments, instead of the more dynamic actin cytoskeleton (Vasioukhin, Bauer et al. 2000).

Tight junction components are initially transported to the cadherin positive cell-cell contact sites, but as the junction matures they re-localize to form distinct more apical junctions. Tight junctions do similar to adherens- and desmosome junctions consist of trans-membrane proteins (claudins, occludins, tricellulin, Junctional Adhesion Molecules (JAMs) or Coxsackievirus and Adenovirus Receptors (CARs)). Alike the cadherins, the cytoplasmic domain of these proteins is connected to the actin cytoskeleton (Steed, Balda et al. 2010).

Gap junctions are quite different to the other junctional types, as they consist of aggregates of connexin-based intercellular channels. The channels allow transfer of ions and small molecules, needed for transmission of chemical- or electrical signals over a multicellular sheet (Wei and Huang 2013).

Cell-ECM contacts

Similar to how tight- and adherens junction transmembrane proteins link the actin cytoskeleton of two neighboring cells, the integrin receptors connect the actin microfilaments to the ECM. The connection to the underlying matrix, allows the cell to sense, adapt- and respond to changes in the environment. The ECM is much more to a stable cellular sheet, than just a scaffold providing mechanical support. On the contrary, the interface between the cell and its underlying matrix, is an important site of signal transmission (Alenghat and Ingber 2002). At the basal cell membrane the integrin receptors aggregate into Focal Adhesions (FAs), where further actin-binding proteins (talins, filamins, α-actinin, tensin and vinculin etc.) help to establish the connection to the cytoskeleton (Schwartz 2001). Different integrin subtypes show specificity to different ECM components, and their activation results in induction of different signaling cascades or actin filament organization. Furthermore, mechanical force from the ECM can be applied to the FAs and through the actin-binding proteins induce re-organization of the actin cytoskeleton (Ciobanasu, Faivre et al. 2013).

In some epithelia, where there is an increased demand for resistance to mechanical forces and hence a particular need for rigid connections to the ECM, the cells also connect to the ECM through specialized integrins linked to intermediate filaments. Such junctions are named hemidesmosomes, and similar to desmosomes they provide a more firm connection compared to corresponding actin-based junctions (Litjens, de Pereda et al. 2006). Through the integrin-based contacts the ECM can communicate signals to the cells, which through cytoskeletal remodeling can result in cell growth, -death (apoptosis), -differentiation or -migration (Ingber 2002).

Cell polarity

Cell polarity is referring to the asymmetrical distribution of cell contents like lipids, proteins, organelles (specialized structures/ organs of the cell) or cytoskeleton etc. (Bryant and Mostov 2008). Cells can be polarized in a front-rear or top-bottom (apical-basal) fashion, or across a multicellular epithelial plane (planar cell polarity). Front-rear polarization is mainly implemented during directional cell migration towards a pro-migratory cue. Planar cell polarity is established to organize and coordinate direction of both subcellular- (hair bundles in sensory neurons of the inner ear) and multicellular structures (hair follicles of the skin). During tissue morphogenesis, planar cell polarity is playing an important role in regulation of tissue elongation (Zallen 2007).

Apical-basal polarity has been recognized as an essential cellular event driving tubulogenesis and functionality of many organs. It allows for specialization of different membrane domains and further directional vesicle transport needed for localized secretion and absorption. Apical-basal polarization requires coordination of signals

received basally from the ECM and laterally via cell-cell junctions, as well as lack of matrix signals from the apical membrane domain (O'Brien, Zegers et al. 2002). Integrins in connection with matrix proteins have been shown to be responsible for the initial polarization cue. Secondly, a signal from the Phosphatase and Tensin homolog (PTEN) at the cell-cell junction is causing separation of lipids of the cell membrane, to define the apical- and basolateral membranes, respectively (Rodriguez-Fraticelli, Galvez-Santisteban et al. 2011). The different membrane compositions cause further recruitment of membrane domain specific proteins. Some of the first proteins to be transported to the apical membrane are Crb3 and Par3, further recruiting Pals1/Patj and Par6/aPKC, respectively. Together with the Rho GTPase Cdc42, these proteins define the apical membrane. The composition of the basolateral membrane attracts a different set of proteins (Scribble, Dlg, Lgl, Par1 and Par5). The specific localization of the apical- and basolateral protein complexes is regulated by mutual negative control. For example the Atypical Protein Kinase C (aPKC) can phosphorylate Lgl and Par1 causing their exclusion from the apical membrane domain. Similarly, aPKC can following the initial apical membrane formation, through phosphorylation, restrict Par3 expression to apical cell-cell adhesions. Following the re-localization, Par3 acts to promote adherens junction formation, by enrolling cadherins and their associated catenins (Tepass 2012). The directional transport delivering vesicles specifically to apical- or basolateral cell membranes is dependent upon a close interaction with both the microtubule- and actin cytoskeleton. In addition, especially the actin cytoskeleton plays a crucial role at the apical membrane during early tubulogenesis (Rodriguez-Fraticelli, Galvez-Santisteban et al. 2011).

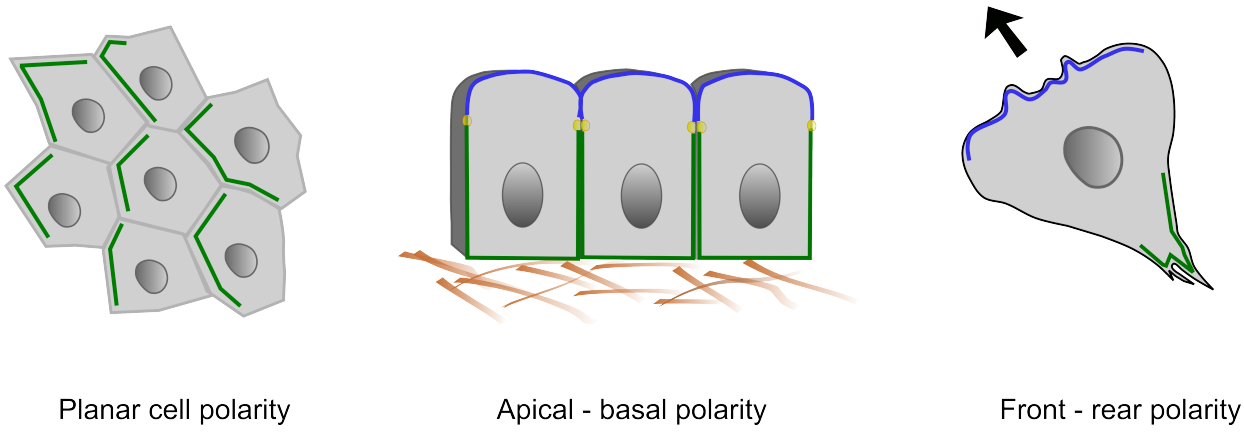

Figure 2. Different types of cell polarity control diverse cellular processes. Planar cell polarity is used to organize and synchronize the direction of subcellular/ multicellular structures, apical-basal polarity enables directional transport and spatially restricted absorption and –secretion, front-rear polarity allows for directional migration.

Sorting and targeting of membrane proteins

For a cell to polarize in an apical-basolateral fashion, correct sorting of membrane proteins to the different membrane domains (e.g. apical and basolateral) is crucial. In order for such sorting and transportation to function, a tight coordination of intracellular- and external processes is required eg. intrinsic sorting codes, segregation of membrane components to distinguish the different domains and receptors sensing connections to neighboring cells as well as underlying ECM. Once synthesized in the endoplasmic reticulum (ER), membrane proteins usually have to pass through several

cellular compartments (e.g. ER, Golgi and endosomes) to reach their final destination. To get from one compartment to the next, the proteins are packed into coated vesicles. The proteins coating the vesicles differ depending on the target site of the transport, COPII for pre-Golgi transport and COPI for intra-Golgi transport etc. In turn, vesicle tethering and fusion at the target site can be regulated by Rab GTPases and SNAREs, respectively (Mellman and Nelson 2008). SNAREs are protein complexes expressed on both vesicles and target membranes. The composition of the complex specifies transport to the apical or basolateral membrane domain, respectively. Rab GTPases cycle between inactive (GDP bound) or active (GTP bound) configurations, regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The TRAPPII protein complex is a GEF activating the Rab1 GTPase, allowing it to recruit its effectors, and ultimately (through SNARE interactions) causing fusion of the vesicle with the target membrane. In mammals, the Rab1-TRAPPII protein complex controls tethering of COPI coated vesicles within Golgi (Barrowman, Bhandari et al. 2010).

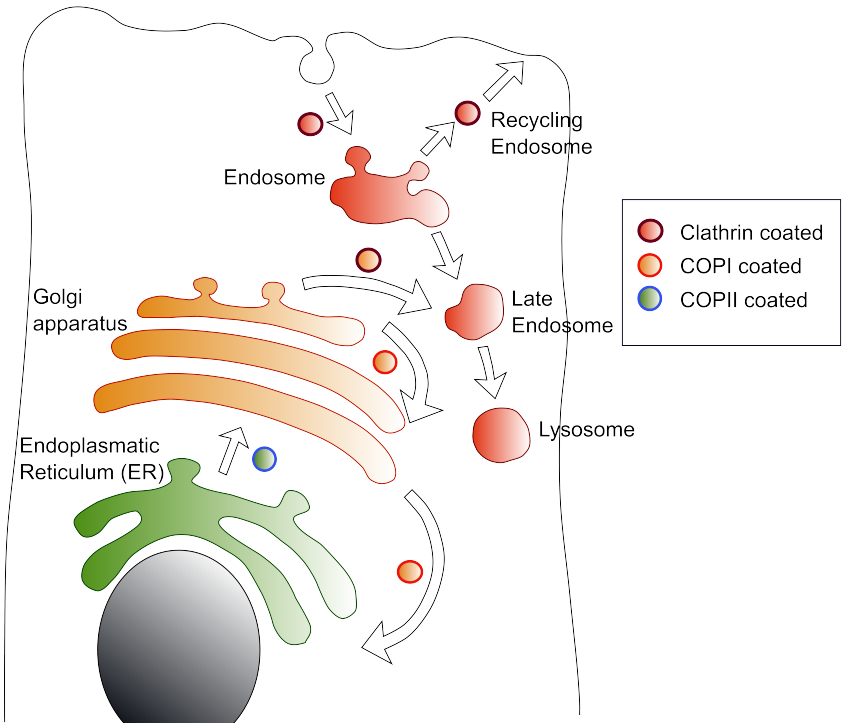

Figure 3. Vesicular transport allows proper targeting of membrane proteins. Different vesicular coating in combination with distinct protein complexes at the target membrane, allows for specific protein transport to apical- or basolateral membrane domains, respectively. Vesicles transporting cargo from the Endoplasmatic Reticulum (ER) to the Golgi apparatus are coated with COPII-, while vesicles trafficking within the Golgi apparatus are coated with the COPI protein complex. TRAPPII is part of the regulatory machinery, controlling vesicle tethering of COPI vesicles within the Golgi apparatus.

The cytoskeleton

The cell cytoskeleton is a dynamic network consisting of actin microfilaments, microtubule and intermediate filaments. As mentioned in preceding paragraphs, the cytoskeleton in many ways is involved in initiation of cell-cell contacts as well as establishment of apical-basal polarity.

Microtubule

An organized microtubule cytoskeleton is crucial for proper directional transport and sorting of apical- and basal proteins, as well as for correct localization of the mitotic spindle, promoting asymmetric- or symmetric cell divisions.

Microtubule filaments are polymers of α- and β-tubulin, which are intrinsically polarized with a plus- and a minus-end. In an un-polarized cell the net microtubule polarization is null, since the minus ends are attached to the centrosome at the cell center and the plus ends are directed towards the cortex. Upon cell-cell interactions, the microtubule organization is altered and instead aligned along the apical-basal polarity axis, with the minus ends facing the apical- and the plus ends the basal domain. Microtubule filaments are used as tracks for vesicle transport. Motor proteins are moving along the fiber towards either the plus- or minus end, allowing for directional protein transport (Musch 2004, Bergstralh, Haack et al. 2013).

Intermediate filaments

Intermediate filaments mainly serve to protect the cell from mechanical stress. In epithelia, intermediate filaments are connected to specialized cadherins of desmosomes, while they in endothelia associate to adherens junctions.

The composition of intermediate filaments are much more diverse compared to the structure of the other cytoskeletal subtypes (Keratins in epithelia, Vimentin in endothelia, mesenchymal- and hematopoietic cells, Desmin in muscle cells). In epithelial-derived cancer cells the presence of vimentin intermediate filaments is a marker of Epithelial-To-Mesenchymal (EMT) transition, correlating with enhanced migratory and invasive capacity and poor prognosis (Gruenbaum and Aebi 2014).

Actin cytoskeleton

As already mentioned, the actomyosin network is coupled to tight- and adherens junctions, but also to focal adhesions connecting the cell to the underlying ECM (Gorfinkiel and Blanchard 2011). Cell-cell junctions can be connected to different types of actin structures, namely cortical or circumferential actin and radial actin filaments, respectively. The cortical actin stabilizes the junction, while the radial actin filaments have been shown to act in the opposite manner to induce weakening and opening of the junction, for example as a response to an inflammatory stimuli (Millan, Cain et al. 2010).

Actin filaments are spontaneously polymerized from globular actin monomers (Pollard and Cooper 2009). However, since the actin cytoskeleton is involved in many diverse cellular processes (cell shape changes, cell migration etc.), it is of great importance for actin filament polymerization to be strictly regulated in space and time. Such stringent control is ensured by nucleation complexes (eg. Arp2/3 complex and formins), acting downstream of small Rho GTPases. The rate of polymerization can be determined by actin-binding proteins controlling localization of actin monomers, actin filament capping, disassembly of old filaments contributing to the pool of monomers and stabilization through cross linking of filaments. Rho GTPase activity is regulated through binding to GTP (active) or GDP (inactive), respectively. Cdc42, Rac1 and RhoA are the most studied small GTPases. They are all in various ways in control of actin polymerization. Cdc42 and Rac1 play important parts during cell migration, for establishment of lamellipodia and filopodia, respectively. RhoA has been quite extensively studied as a regulator of contractile actin filaments associated to focal adhesions. The contractility is mediated through coupling of the actin filaments to non-muscle myosin II, a motor protein complex moving along the microfilaments (Ridley,

Schwartz et al. 2003). As discussed in more detail below, actomyosin contractility is a central mechanism during tissue morphogenesis and tubulogenesis.

The role of actomyosin contraction in tissue morphogenesis

Intercellular linkage of contractile actin filaments, allows morphological alterations of individual cells to be synchronized to further promote creation of novel tissue level structures; infolding, elongation etc.. Coordinated cell shape changes within a polarized multicellular sheet, are crucial for tubulogenesis and organogenesis. Hence, there is a tight correlation between cell geometry and tissue topology (Gibson and Gibson 2009). For example, tissue invagination during tube formation is dependent upon apical constriction, where the apical membrane is shrinked to create bottle-shaped cells. This is achieved through apical recruitment of contractile actomyosin filaments. In addition, the mechanism of apical constriction is used during cell extrusion and wound healing. Tissue elongation is another central process during embryogenesis, enabling growth of the developing embryo. This process is also highly dependent upon actomyosin driven tissue re-organization, for example through intercalation where cells exchange positions/ neighbors without involving cell migration. Instead, intercalation is dependent upon abundant junctional remodeling, driven by actomyosin-mediated contraction, as well as planar cell polarity.

The same mechanism of specified junctional growth/ -shrinkage also occurs during packing of cells within an epithelial sheet (Pilot and Lecuit 2005). Already in the early 20th century it was observed that epithelial cells, due the minimal surface energy and tight packing, obtained hexagonal shapes when organized into a monolayer. The polygonal distribution of cells was observed to be nearly constant in between tissues and further species, with most of the cells presenting as hexagons, followed by pentagons. The constant distribution of geometrical shapes, is believed to stem from a combination of an equilibrium obtained through cell divisions, followed by active junctional rearrangements (Gibson and Gibson 2009).

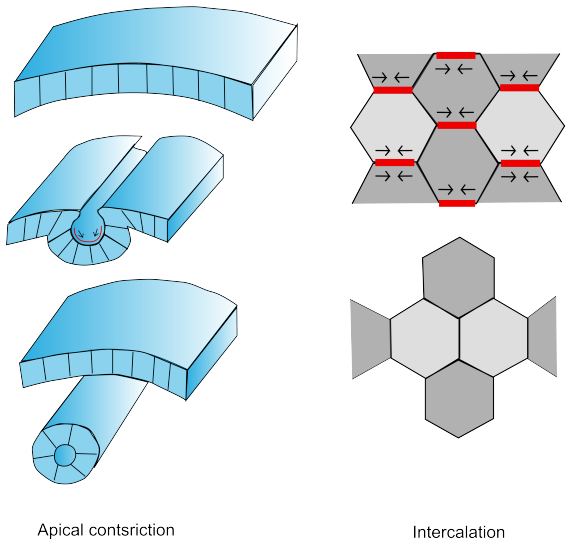

Figure 4. Synchronized cell shape changes drive tissue level morphological transformations. Apical constriction and intercalation are both processes highly dependent on actomyosin-mediated cell shape changes. Actomyosin contractions (red, arrows) at the apical membrane and cell-cell junctions, respectively, result in development of new tissue structures.

Tubulogenesis

As discussed above, formation of most organs require further modification of the multicellular sheet, often aiming to form a final tubular structure e.g. kidneys, intestines, lungs and vascular system. Tube formation requires high level of intercellular coordination, and several different modes of tubular development have been observed e.g. wrapping in vertebrate neural tube formation, budding throughout mammalian lung formation, cavitation during salivary gland morphogenesis, cord hollowing during Drosophila heart tube formation and lastly cell hollowing (Lubarsky and Krasnow 2003). Much of the knowledge regarding the mechanisms underlying tube formation has been gained through studies in the Drosophila- or Zebrafish model systems, or from three-dimensional models *in vitro*. The function of apical membrane proteins (Crumbs), cytoskeletal regulators (Rac GTPase) and cell-cell junction proteins (Claudin) have all been proven crucial for different steps of Drosophila salivary gland invagination and –tracheal tube expansion, respectively (Myat and Andrew 2002) (Chihara, Kato et al. 2003, Wu, Schulte et al. 2004). Studies in the zebrafish model have been providing evidence for the importance of the aPKC polarity protein in tubulogenesis of both the neural tube and digestive tract (Horne-Badovinac, Lin et al. 2001). In additional, the final steps of lumen expansion are more dependent upon pressure, created through apical secretion of matrix proteins and –ions, as well as accumulation of fluid (Tsarouhas, Senti et al. 2007) (Bagnat, Cheung et al. 2007).

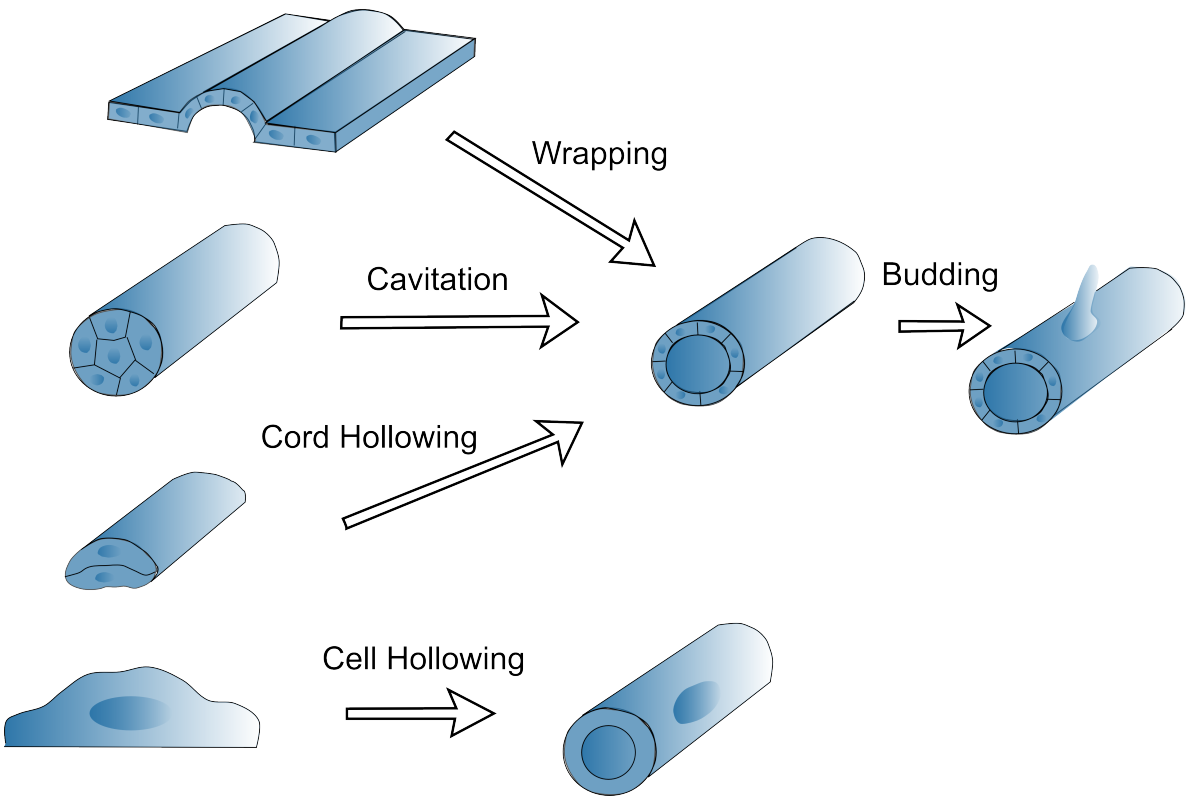

Figure 5. Different modes of tubulogenesis. Several different mechanisms of tube formation have been observed in different model systems and –organs. Modified from Lubarsky, B. and Krasnow, MA., 2003. *Tube morphogenesis: making and shaping biological tubes.* Cell 112, 19-28.

Vascular development

The cardio-vascular system is the first organ to develop during embryogenesis. Delivery of oxygen and nutrients, as well as removal of carbon dioxide and metabolic waste products becomes crucial as soon as the embryo grow beyond the size allowing for passive diffusion (Chung and Ferrara 2011). The vascular tubes are highly specialized structures, where large tubes allow for fast and efficient transport and smaller tubes with slower flow, are optimized for gas or nutrient exchange between the blood and surrounding tissue. Vascular tubes of different sizes and organ locations are all formed either through cord hollowing during formation of the initial vascular plexus (vasculogenesis), or later on through budding during vessel remodeling (angiogenesis).

The tubes of the vertebrate vascular system are lined with endothelial cells, and further stabilized by mural cells e.g. vascular smooth muscle cells (vSMCs) or pericytes. The large arteries and veins are divided into three different layers; tunica intima (endothelial cells), tunica media (smooth muscle- and nerve cells, as well as extracellular elastin) and tunica externa (collagen fibers and fibroblasts) (Lammert and Axnick 2012). The walls of the veins are thinner compared to corresponding arteries, reflecting the different magnitudes of pressure-exposure from the circulating blood. Microvessels (capillaries, postcapillary venules and terminal arterioles) are composed of even thinner walls consisting of a single endothelial layer, covered and supported by pericytes. The endothelial cells and the pericytes share the basement membrane (BM), in which the pericytes are embedded.

Pericytes stabilize vessels, control permeability, and are further responsible for constriction of capillaries in response to vaso-constrictive compounds. Pericytes and endothelial cells are in direct contact through holes in the basement membrane, either through peg-socket type adhesions or adhesion plaques. The adhesion plaques are similar to adherens junctions in between adjacent endothelial cells, and consequently are in contact with the actin cytoskeleton (Gaengel, Genove et al. 2009, Armulik, Genove et al. 2011).

The actin cytoskeleton is involved in most steps of vascular tube formation, from progenitor cell migration, to coordination of cell shape changes, force generation during lumen expansion, and generation of contractile pulling forces in the tip-cell of a migrating angiogenic sprout (Strilic, Kucera et al. 2009) (Helker, Schuermann et al. 2013) (Franco, Blanc et al. 2013).

Vasculogenesis

During embryonic development in mammals, the first hematopoiesis as well as vasculogenesis (*de novo* formation of blood vessels), takes place in the yolk sac where blood islands are formed as early as embryonic day 6.5 (E6.5) (Drake and Fleming 2000, Chung and Ferrara 2011). The progenitor cells of the blood islands give rise to hematopoietic cells, as well as the yolk vasculature and on the embryonic side; the dorsal aortae, vitelline arteries and –veins and endocardial tube (De Val and Black 2009).

During formation of the first intra-embryonic tubes, the dorsal aortae, the angioblast progenitor cells migrate from the lateral plate mesoderm to the embryonic midline, where they align to form a multicellular cord. The midline migration is believed to be guided by growth factors such as VEGF-A and sonic hedgehog (Chung and Ferrara 2011). Once aligned, the progenitor cells elongate to form immature endothelial cells and further start to contact their neighbors. Even at this early stage, the cell-cell contacts are VE-cadherin positive. VE-cadherin further induces expression of glycoproteins and

actin polymerization at the apical membrane. The repellant negative charge of the apical glycoproteins in combination with the actomyosin generated force, allow for separation of the two opposing apical cell membranes, to generate a small initial lumen (Lammert and Axnick 2012). Once formed, the aortic lumen can be further expanded by cell proliferation, as well as shear stress produced upon onset of circulation (Strilic, Kucera et al. 2009).

VEGF-A is involved in both the guided midline migration of progenitor cells and further in stimulation of the actomyosin contraction needed to separate the apposing apical surfaces. The impact of VEGF-receptor signaling during vascular development is emphasized by studies in mice, where targeting of a single VEGF-A allele results in embryonic lethality and abundant vascular defects (Carmeliet, Ferreira et al. 1996, Ferrara, Carver-Moore et al. 1996). A number of other genes with known functions within cell-ECM adhesion, cell-cell adhesion, apical-basal polarity and actin cytoskeleton regulation have also been genetically deleted in mice. The results clearly show the importance of all of these processes and further their interconnection during aortic tube formation. For example, aortic endothelial cells depleted of β1-integrin failed to correctly localize polarity- as well as cell-cell adhesion proteins, resulting in aortic constrictions and embryonic lethality (Zovein, Luque et al. 2010). Another interesting study could show Rasip1, an effector of the small GTPase Rap1, and its associated GAP to be crucial for proper actin cytoskeleton organization during aortic lumen formation. Rasip1 finely balanced the expression levels of the small GTPases Cdc42/ Rac1 and RhoA, and in its absence the elevated RhoA-mediated actomyosin contractility resulted in aortic constrictions and embryonic lethality (Xu, Sacharidou et al. 2011). As discussed in more detail below, also depletion of VE-cadherin resulted in aberrant aortic development caused by defects in endothelial cell apical-basal polarity and actomyosin contractility (Strilic, Kucera et al. 2009).

Angiogenesis

Expansion of the vascular plexus is accomplished through angiogenic sprouting from pre-existing vessels. The new sprouts consist of a mixture of tip- and stalk cells. As the name suggests, the tip-cell is located at the front of the sprout, where it extends actomyosin rich filopodia. The filopodia on the frontal tip-cell function to scan the environment for potential pro-migratory cues (VEGF-A etc.), as well as pull the sprout forward. The stalk-cells on the other hand proliferate- and organize to create a central lumen. The specification into tip- and stalk cells respectively, is a highly dynamic process regulated by VEGF-A- and Notch signaling. Upon binding to the VEGFR-2 receptor, VEGF-A induces tip-cell specification, while Notch signaling promotes stalk-cell fate and appurtenant proliferative mode. By lateral inhibition, cells adapting the tip-cell fate, prevents neighboring cells from receiving VEGF-A stimulation and instead drive them into adapting stalk-cell properties (Geudens and Gerhardt 2011).

Newly formed sprouts can make contact with each other, or pre-existing vasculature, through anastomosis. The contact is initiated in between filopodial extensions of the tip cells of two adjacent vessels. Studies of the zebrafish cranial vasculature have revealed a stepwise mechanism being responsible for the fusion; involving VE-cadherin dependent cell-cell contact formation, *de novo* polarization, apical membrane invagination and cell remodeling (Lenard, Ellertsdottir et al. 2013). Interestingly, macrophages have been ascribed a role in bridging the extended filopodia of apposing tip cells (Fantin, Vieira et al. 2010).

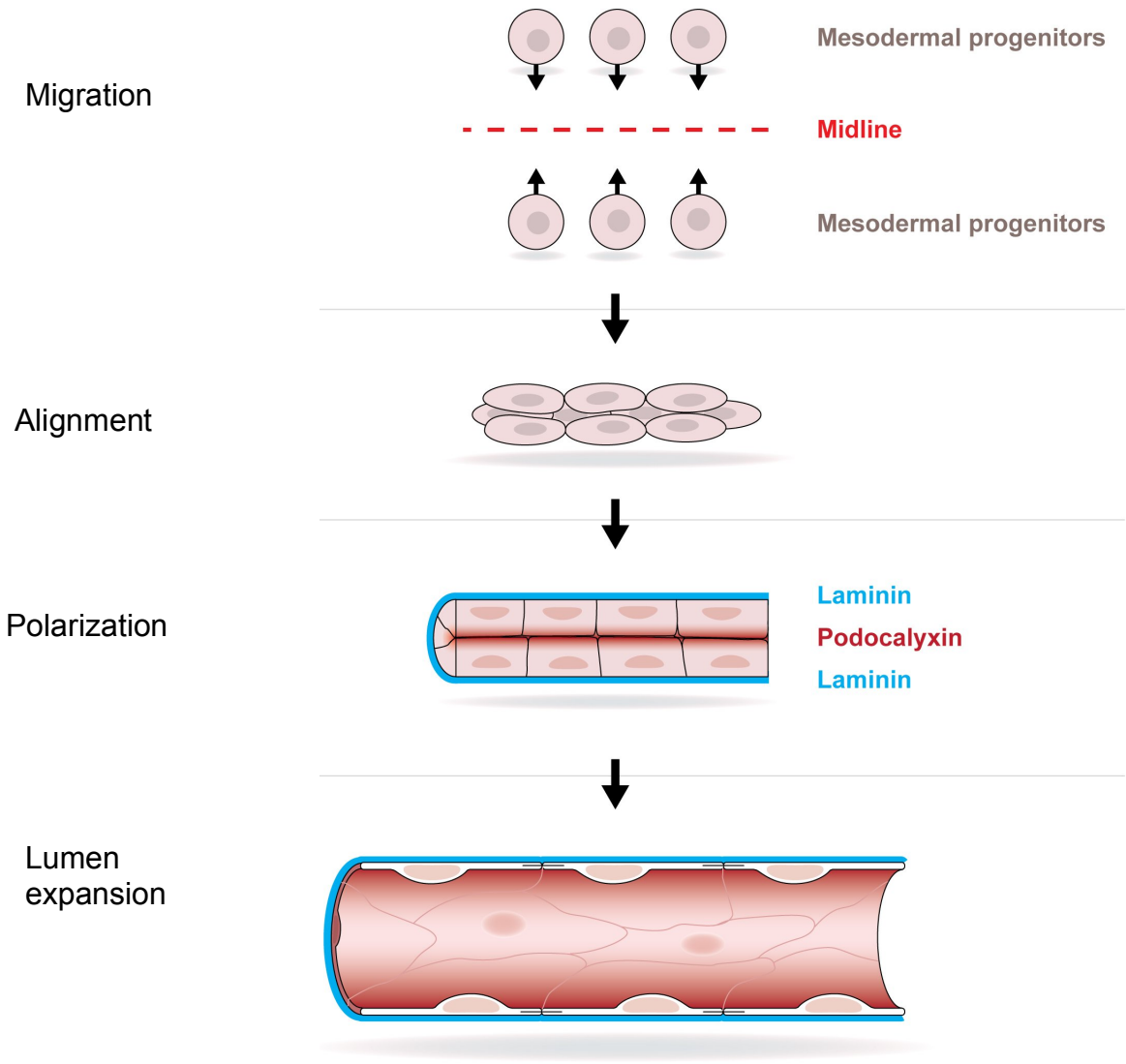

Figure 6. The steps of vascular tube formation. Angioblast progenitor cells migrate to the midline where they align and elongate to form immature endothelial cells. Following the alignment, cell-cell contacts are initiated and apical-basal polarity established. Negatively charged glycoproteins in collaboration with actomyosin-generated forces, enable separation of the two opposing apical membranes, resulting in formation of a lumen.

Adapting arterial- or venous fate?

The perception of how endothelial cells differentiate and how their contribution to arteries or veins are controlled, has the last decades changed dramatically. The former idea that solely environmental factors such as shear stress and flow determined the fate of endothelial cells has now been rejected (De Val and Black 2009). This notion was contradicted by transplantation studies in the quail embryo, demonstrating that embryonic endothelial cells at E7 had lost their capacity to indiscriminative contribute to arterial- and venous vessels. Hence, the endothelial cells must have adapted properties specific for arteries or veins, respectively. Consequently, analysis of molecular markers has now revealed different molecular signatures of arterial- and venous endothelial cells (EphrinB2/EphB4, Notch/Dll4, VEGFR2/VEGFR3 etc.), already

prior to onset of flow (Lawson and Weinstein 2002). From studies in the zebrafish, it has become evident that arterial- and venous progenitors differentiate already before migration to the midline. The arterial progenitors were observed to migrate first, followed by a second wave of migration, by cells contributing to the developing vein. As the migration of progenitor cells was shown to be guided by VEGF-A, a relatively higher exposure to VEGF-A was suggested to cause the cells located most medial at the lateral plate to adapt an arterial fate. Subsequently, cells at more distal locations would be expected to take part in the second wave of migration and hence contribute to development of the vein (Lawson and Weinstein 2002, Kohli, Schumacher et al. 2013).

Lymphatic vessels

Following the arterio-venous specification, a subpopulation of venous cells continues to differentiate into lymphatic endothelial cells (LECs). Groups of cells bud of from veins to form lymph sacs, further differentiating into collecting lymphatic vessels or lymphatic capillaries. Prox1 and VEGF-C/VEGFR-3-signaling are factors responsible for the differentiation and migration, respectively (Yang and Oliver 2014). The lymphatic system is important for draining extra-vasated fluid and macromolecules from the surrounding tissue, transporting immune cells and absorbing lipids from the gut. The lymphatic capillaries described above, are the sites of fluid absorption. These are blunt-ended vessels constructed out of endothelial cells, but lacking mural cell coverage and probably basement membrane, as well. LECs are differently shaped compared to endothelial cells of blood vessels. They hold the shape of an oak-leave, and their cell-cell junctions, even though VE-cadherin-based, are much more discontinuous (Martinez-Corral and Makinen 2013).

VE-cadherin in vascular development and permeability

As already mentioned, VE-cadherin contributes to vascular lumen formation through initiation of endothelial cell-cell contacts, and recruitment of a contractile apical actomyosin network (Strilic, Kucera et al. 2009). In addition, VE-cadherin acts as a key regulator of permeability and leukocyte diapedesis in mature vessels (Wessel, Winderlich et al. 2014). Furthermore, VE-cadherin stabilizes angiogenic sprouts and contributes to anastomosis, limiting vascular sprouting. The severe effects of VE-cadherin silencing have been studied in mice and zebrafish, where its deletion was shown to cause abundant hemorrhage, luminal constrictions and hypersprouting (Carmeliet, Lampugnani et al. 1999, Montero-Balaguer, Swirsding et al. 2009). The cytoplasmic domain of VE-cadherin associates to p120-catenin and β-catenin/ plakoglobin. As β-catenin and plakoglobin, when unbound, can transport into the nucleus to promote-/ repress transcription of specific target-genes, VE-cadherin functions as an important transcriptional regulator. For example, in the absence of VE-cadherin, nuclear β-catenin represses expression of the tight junction protein claudin-5 (Giannotta, Trani et al. 2013). β-catenin/ plakoglobin in turn binds α-catenin, traditionally believed to link VE-cadherin to the actin cytoskeleton. Recently, its role as a linker-protein has been questioned, as different monomer/ homodimer configurations are needed for the interaction with actin filaments and β-catenin, respectively (Yamada, Pokutta et al. 2005). Several other actin-associated proteins bind α-catenin (vinculin, EPLIN etc.) and one of them can potentially act as the missing link, coupling the cadherin junctions to the actin cytoskeleton (Peng, Maiers et al. 2012) (Abe and Takeichi 2008). p120-catenin stabilizes VE-cadherin at the junction, and prevents its internalization (Xiao, Allison et al. 2003). Furthermore, the stability of VE-cadherin appear to depend on its phosphorylation-state, which interestingly has been shown to differ in between

arterial- and venous endothelial cells, possibly due to differences in shear stress exposure (Orsenigo, Giampietro et al. 2012).

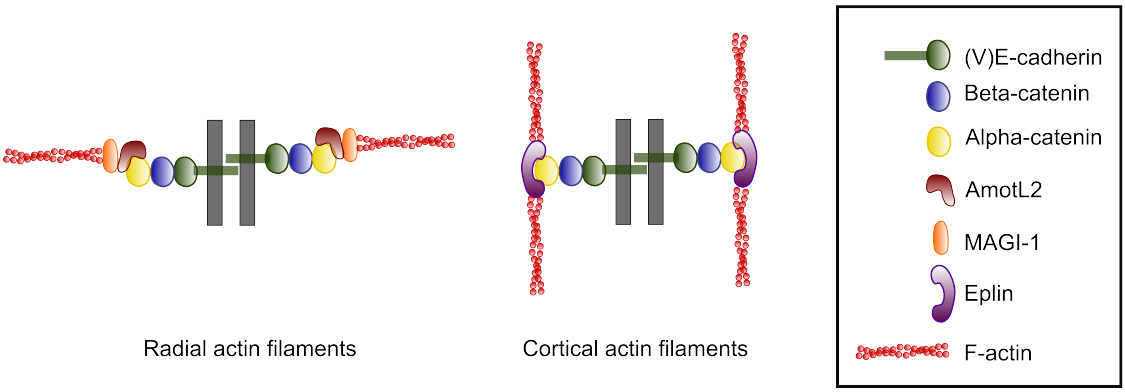

Figure 7. Intracellular catenins connect VE-cadherin to the actin cytoskeleton. The intracellular domain of VE-cadherin associates to β-catenin, further connected to α-catenin. Additional linker proteins are needed for the association to actin filaments. Different proteins may show specificity to different actin structures, eg. radial- and cortical actin filaments, respectively.

Junctional force sensing and –transmission

Mechanical forces are perceived by the cell and translated into intracellular biochemical signaling cascades, through a process called mechanotransduction. The cellular ability to rapidly sense and respond to extracellular mechanical signals is of particular importance in tissues constantly exposed to shearing forces, like blood vessels, muscles, bones and cartilage. Defects in sensing/ relaying such signals can result in pathologies; including hearing loss, lung dysfunction, muscular dystrophies, atherosclerosis and cancer (Gulino-Debrac 2013).

As discussed in the previous paragraph, α-catenin can probably not alone vouch for the connection of the cadherin-based adherens junctions to the actin cytoskeleton, instead it must recruit other actin-associated proteins. One of the candidates; vinculin, was recently found not to be essential for junction establishment or association of cadherins to the actin cytoskeleton, but to modulate junction integrity as a response to mechanical force or growth factor stimuli (le Duc, Shi et al. 2010, Huveneers, Oldenburg et al. 2012). Interestingly, vinculin could only be observed in association with α-catenin upon force exposure. Consequently, pulling forces from neighboring cells or intracellular actin filaments, caused α-catenin to change conformation, which exposed the otherwise masked vinculin-interaction-site (Yonemura, Wada et al. 2010). Upon its association, vinculin stabilizes the junction, counteracting the pulling/compressing force (through recruitment of actin polymerization factors etc.) (Leerberg, Gomez et al. 2014). In addition, there is evidence for the nature of the α-catenin-actin filament binding to change and be strengthened, as a response to the force-induced conformational changes (Engl, Arasi et al. 2014). Similarly, force-mediated conformational changes of the integrin-associated protein talin at the focal adhesion, expose previously masked vinculin-binding sites. Association of vinculin to the focal adhesion results in downstream re-organization of FA-associated actin filaments (del Rio, Perez-Jimenez et al. 2009). These findings reveal a role of adherens junctions and focal adhesions as active sensors-, in contrast to passive transmittors-, of environmental force alterations (Yonemura, Wada et al. 2010).

Mechanotransduction in blood vessels

All endothelial cells are subjected to shearing forces from the circulating blood, but the stress is not homogenously applied over the circulatory system. On the contrary, it varies heavily, with the arteries being exposed to much higher stress compared to veins, due to higher blood pressure (Gulino-Debrac 2013). As the heart releases blood in a pulsatile fashion, the arteries experience pulses of increased blood pressure. To counteract this elevation in pressure, endothelial cells signal to the surrounding smooth muscle cells, to cause relaxation and subsequent vessel expansion. Also within arteries, the shear stress applied to endothelial cells varies between areas of straight- and bent vessel segments/areas of bifurcations. Endothelial cells at vessel bifurcations are subject to fluctuating shear stress, causing turbulence. Even in healthy individuals, areas of turbulent flow are sites of mild chronic inflammation, which can if combined with additional risk factors (diabetes, obesity, hyperlipidemia, smoking, sedentary lifestyle etc.) ultimately cause development of an atherosclerotic plaque. As the plaque grows/ ruptures it can obstruct-, or in the case of thrombus formation completely block the vessel, causing myocardial infarction or stroke.

Several different mechanisms of how endothelial cells detect alterations in flow/ shearing forces have been suggested. For example, the apical glycocalyx, primary cilia, ion channels, integrins and cell-cell adhesion proteins (Hahn and Schwartz 2009). All of these mechanisms involve some level of force-induced conformational/ organizational changes, at the molecular- or structural level. The actin cytoskeleton in connection with both the ECM and neighboring cells, can mediate flow-induced endothelial cell re-arrangements. More specifically, VE-cadherin has been shown to form a mechanosensory complex, together with VEGFR-2 and the Platelet Endothelial Cell Adhesion Molecule (PECAM-1), resulting in integrin activation, actin filament organization and alignment of endothelial cells to flow (Tzima, Irani-Tehrani et al. 2005).

Mechanotransduction in tumor development

Mechanotransduction also has a big impact for development of certain diseases, for example cancer. Cancer research has focused mostly on the effects of the force transmitted between tumor cells and the surrounding ECM, even though indirectly affecting also the tension applied to cell-cell junctions (Maruthamuthu, Sabass et al. 2011). There is a tight correlation between stiffness of a tissue and the risk of both tumor development and –progression. Several diseases involving alterations of the ECM (liver cirrhosis, cystic fibrosis etc.) can predispose the affected tissue to tumor formation. In addition, analysis of matrix stiffness is applied during mammography screening, where detection of a dense breast tissue identifies a population with increased risk of developing breast cancer (Pickup, Mouw et al. 2014). Indeed, mice with a more dense stromal collagen network, showed both increased mammary tumor development and –progression (Provenzano, Inman et al. 2008). Stiff matrix has also been shown to interfere with organized lumen formation and to induce invasive- and migratory behavior *in vitro*, through disruption of apical-basal polarity (Schedin and Keely 2011). Furthermore, collagen fibers of the ECM can function as tracks for migratory cells. Hence, abundant collagen deposition facilitates tumor cell invasion into surrounding stroma and further blood- and lymphatic vessels (Shields, Fleury et al. 2007, DuFort, Paszek et al. 2011).

The enhanced migratory potential, can be linked to actin cytoskeletal remodeling. Increased matrix stiffness elevates the force load applied to cellular focal adhesions. To

compensate for the alteration in tensional homeostasis, the cell induces aggregation and activation of integrins and other focal adhesion associated proteins, resulting in activation of the small GTPase RhoA and consequent re-organization of the actin cytoskeleton. In addition with relevance for tumor formation, focal adhesion activation induces pro-proliferative signaling (DuFort, Paszek et al. 2011).

Mechanotransduction through the Hippo-signaling pathway

The signaling activity of the hippo-pathway is commonly altered in some types of cancer, for example liver and prostate. Recently, the Hippo-pathway received a lot of attention for being regulated by external mechanical forces (Zhao, Wei et al. 2007). Proper control of this signaling pathway has previously been shown to be crucial for organ development, as its deregulation can affect both organ growth and cell differentiation. The Hippo-pathway consists of MST- and LATS-kinases, controlling the activity of the transcriptional co-activators YAP and TAZ. Phosphorylation of YAP/TAZ by the upstream kinases, results in their retention in the cytoplasm or at the cell-cell junction, preventing activation of target gene transcription. As many of the target genes are pro-proliferative or anti-apoptotic respectively, YAP/TAZ activation results in tissue growth. During physiological conditions, YAP/TAZ activity is limited by contact inhibition of proliferation, a mechanism allowing cells to control their proliferative rate as a response to formation of cell-cell contacts. Recently, the contact inhibition of YAP/TAZ-activity, was shown to be dependent on contact-induced changes in the actin cytoskeletal architecture. Furthermore, culturing cells on soft matrix, known to cause a decreased cell area and reduction of actin filaments, resulted in inactivation of YAP and TAZ. Exactly how this regulation is executed is not yet completely known, and both LATS-dependent and –independent pathways have been suggested (Dupont, Morsut et al. 2011) (Aragona, Panciera et al. 2013). Indeed, the Hippo-signaling pathway can be one way to explain the correlation between dense tissue and tumor progression. Importantly, YAP-expression has been shown not only to be upregulated in cancer cells, but also in cancer-associated fibroblasts (CAFs) of the tumor stroma. CAFs can upon YAP-activation further promote matrix stiffening and tumor progression (Calvo, Ege et al. 2013).

Tumor microenvironment

As mentioned in previous paragraphs, the tumor stroma can indeed contribute to tumor progression, for example through modulation of ECM stiffness. However, the tumor stroma does not simply consist of matrix proteins, but is also highly colonized by a large variety of cells (eg. Endothelial- and lymphatic endothelial cells, cancer associated fibroblasts and immune cells). Cancer associated fibroblasts (CAFs) can, in addition to their already mentioned ECM-remodeling activity, release cytokines stimulating invasion of immune cells, as well as blood- and lymphatic vessels (Quail and Joyce 2013). Most types of immune cells can be recruited to developing tumors, with varying outcomes. For example, macrophages and neutrophils can be polarized into either type-I or type-II cells, causing more or less the opposite actions. M1 macrophages act in an anti-tumorigenic fashion by releasing pro-inflammatory cytokines and promoting cancer cell apoptosis. M2 macrophages on the other hand, are counteracting the anti-tumorigenic immune responses and further promoting angiogenesis, as well as tumor cell invasion. The switch from M1 to M2, is believed to be induced by hypoxia (Casazza, Di Conza et al. 2014). The tumor stroma can be modified as the tumor progresses, and both macrophages and CAFs have been observed at the invasive front of disseminating tumors, probably releasing pro-migratory and proteolytic factors. In

addition, macrophages can facilitate dissemination of cancer cells into the blood stream (Quail and Joyce 2013).

Tumor hypoxia

Many processes driving tumor progression can be regulated by the oxygenation of the environment. Oxygen levels in tumors are fluctuating over time and in between different tumor areas. Nevertheless, oxygen levels of tumors are in general lower compared to the levels in healthy tissues. Rapid tumor cell proliferation in combination with the abnormal and dysfunctional tumor vaculature, make tumors likely to develop a hypoxic core (Casazza, Di Conza et al. 2014).

As all cells and organs of the human body are dependent on oxygen for energy production, chronic hypoxia normally results in necrosis (cell death). On the contrary, cancer cells have found ways to adapt to the harsh environment. For example, cancer cells to a higher extent use anaerobic metabolism (glycolysis) to fulfill their energy demands. Furthermore, cancer cells can release growth factors, promoting angiogenic growth into the tumor.

Probably the most studied way, by which the cell can respond to hypoxia, is through the Hypoxia Inducible Factors (HIFs). These transcriptional co-activators consist of two components; HIF-1/2α and HIF-1β, with the former being the hypoxia regulated subunits. In the presence of oxygen, post-translational modifications of the HIF-1/2α subunits, allows HIF to interact with the Von Hippel-Lindau (VHL) protein, further causing its degradation. On the contrary, during hypoxic conditions HIF-1/2α is stabilized, and can in complex with HIF-1β bind to hypoxia responsive elements (HREs) in the DNA of target genes, activating their transcription (Harris 2002). Among the target genes are glycolytic enzymes, angiogenic growth-factors, chemokine-receptors, matrix remodeling proteins etc., driving tumor progression (Gordan and Simon 2007). Accordingly, hypoxia has been correlated to aggressive tumors with enhanced metastatic capacity and to poor survival of patients (Hockel, Schlenger et al. 1996).

Tumor vasculature

As a response to the hypoxic environment, tumor cells commonly release pro-angiogenic factors. These growth-factors can be induced through transcriptional activation by HIFs, or through ECM degradation releasing previously bound growth-factor fractions. Endothelial cell detection of the pro-angiogenic cues, induces degradation of the vessel basement membrane to allow for invasion into the tumor (Hanahan and Folkman 1996).

Eventhough, the angiogenic process in tumors mimics its developmental counterpart, tumor vessels are usually highly abnormal, showing excessive sprouting and defective barrier function. The latter could be partly due to decreased pericyte- and irregular basement membrane coverage, but also to alterations in cell-cell adhesion proteins. For example, elevated stromal VEGF-levels cause phosphorylation- and consequent degradation of VE-cadherin. In addition, loss of junctional VE-cadherin leaves the VEGFR2 highly phosphorylated and active, causing sustained stimulation of angiogenesis (Cavallaro, Liebner et al. 2006) (Wallez, Vilgrain et al. 2006).

The increased permeability of tumor vessels, in combination with absence- or low presence of lymphatic vasculature, causes the interstitial fluid pressure to rise. Consequently, oxygen delivery is impaired and the tumor is rendered hypoxic and acidic (Baluk, Hashizume et al. 2005). Furthermore, a high interstitial pressure can be interfering with targeting of the tumor, by increasing drug efflux. Highly permeable tumor vessels also facilitates invasion of immune cells into the tumor, which can further

promote tumor progression, as discussed in previous sections. Intravasation of cancer cells into the blood stream, and extravasation at the secondary site (site of metastasis development), also strongly benefit from a leaky vasculature (Le Guelte, Dwyer et al. 2011).

Tumor progression and –dissemination

Loss of E-cadherin during tumor progression

The processes of tumor cell invasion and –metastasis into blood- or lymphatic vessels and further lymph nodes and distant organs, require plasticity of the epithelial properties. As the tumor develops, it commonly looses characteristic epithelial features like cell-cell and cell-ECM adhesions and apical-basal polarity. Instead the tumor cells adopt new pro-migratory mesenchymal properties, facilitating invasion into adjacent tissues (Kang and Pantel 2013). For example, loss of E-cadherin has been shown sufficient to promote progression of a differentiated (epithelial morphology) benign and non-invasive adenoma, to an invasive malignant carcinoma in the Rip1Tag2 pancreatic cancer mouse model (Perl, Wilgenbus et al. 1998). It seems that loss of E-cadherin cannot by itself initiate tumor growth, but may rather promote progression of already formed tumors.

E-cadherin function can be lost through genetic mutations, or more commonly through epigenetic modifications of the promoter and subsequent transcriptional repression (Jeanes, Gottardi et al. 2008). As already touched up on, loss of E-cadherin can allow nuclear transport of β-catenin. In colorectal cancer where in addition the β-catenin inhibitor Adenomatous Polyposis Coli (APC) is commonly mutated, loss of E-cadherin can result in over-activated Wnt-signaling and uncontrolled cell proliferation (Buda and Pignatelli 2011). Alternatively, E-cadherin function can be compromised through disconnection from the supportive actin cytoskeleton, for example through interference with the Rac1 or RhoA GTPases. Independent of the causal mechanism, loss of E-cadherin function inevitably results in weakening of cell-cell contacts, facilitating tumor cell invasion (Braga, Machesky et al. 1997) (Takaishi, Sasaki et al. 1997) (Sander, van Delft et al. 1998).

Decreased E-cadherin expression in tumor cells is usually accompanied by gain of mesenchymal N-cadherin expression. This cadherin-switch changes the cellular interaction partners from epithelial to stromal cells, and hence promotes invasion into the tumor stroma. The process when epithelial cancer cells adopt more mesenchymal-like features has been named “Epithelial-to-Mesenchymal-Transition” (EMT), and can be induced by signals from the tumor stroma or a hypoxic microenvironment etc. (Yang, Wu et al. 2008) (Moustakas and Heldin 2014). EMT is not a new concept developed by tumor cells. On the contrary, it is crucial for many developmental processes during embryogenesis (gastrulation, neural crest formation etc.). Snail is one of the most well known regulators of EMT (both during development and tumor progression), promoting the mesenchymal phenotype through transcriptional repression of E-cadherin, as well as several apical-basal polarity proteins (Crb3, Patj, Lgl2).

There is evidence for a reversion back to a more epithelial morphology, to be needed for seeding at the secondary site. This process has consequently been named Mesenchymal-to-Epithelial Transition (MET) (Moustakas and Heldin 2014). Colonization at the secondary site can occur immediately after disintegration of cells from the primary tumor. Alternatively, the disseminated cells may stay latent/ dormant (for example in the bone marrow) and not until many years later spread and form a macrometastasis (Kang and Pantel 2013). The awakening from the dormant state can be

induced by internal changes in the cancer cells, alterations in the microenvironment, occurrence of an angiogenic swith or suppression of the immune system (Sosa, Bragado et al. 2014).

Apical-basal polarity in tumor progression – a two sided story

Junctional proteins generally act as tumor suppressors. In contrast, the role of apical-basal polarity proteins in tumor progression is slightly more complex. Loss of the apical polarity protein Par3 was shown to induce invasion and metastasis of breast cancer cells, through weakening of E-cadherin based junctions (Xue, Krishnamurthy et al. 2013). Similarly, altered localization of the basolateral polarity determinant Scribble caused prostate hyperplasia and further tumorigenesis (Pearson, Perez-Mancera et al. 2011). On the contrary, aPKC and Par6, was found to be over expressed in human cancers and the increased levels could be linked to disorganized- and uninhibited growth (Eder, Sui et al. 2005) (Nolan, Aranda et al. 2008). The contradictory results can perhaps be explained by a need for a strict regulation of the balance of apical- and basal polarity determinants, and further their spatial localization. From studies in Drosophila, it became evident that both depletion- and over expression of the apical membrane protein Crumbs resulted in tissue overgrowth and tumor development (Karp, Tan et al. 2008) (Chen, Gajewski et al. 2010).

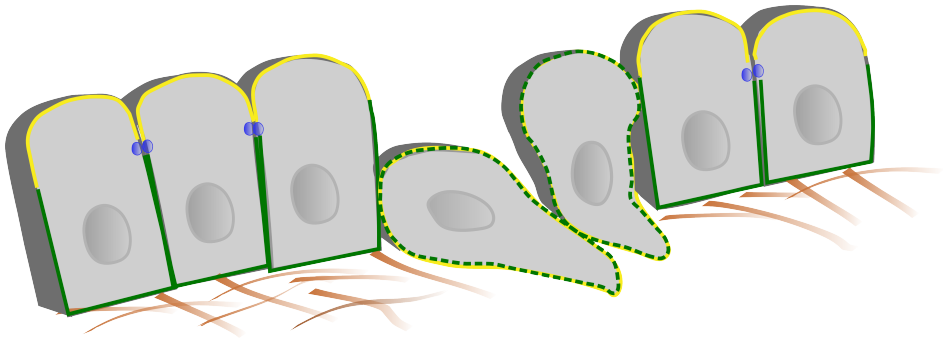

Figure 8. Loss of cell-cell adhesions and apical-basal polarity drives tumor invasion. Typical epithelial features such as cell-cell adhesions (blue) and apical-basal polarity (apical polarity proteins – yellow, basal polarity proteins – green), are commonly lost during tumor progression. Instead, tumor cells adopt mesenchymal properties, facilitating invasion and migration.

The Angiomotin protein family

All the cellular- as well as tissue level processes described in this thesis, has been studied from the point of view of the Angiomotin protein family. The founding member of the protein family (Angiomotin) was initially discovered as being the target of the endogenous angiogenesis inhibitor angiostatin. Consequently, most of the early studies of Angiomotin were performed in endothelial cells with the prospects of impact for anti-angiogenic therapy (Troyanovsky, Levchenko et al. 2001). Two years later, two additional members with similar structural organization was found, and hence named Angiomotin Like-1 and -2, respectively. The two novel family members lack the angiostatin-binding domain, but share with Angiomotin the N-terminal glutamine rich-, coiled-coil- and C-terminal PDZ-binding domain (Bratt, Wilson et al. 2002) (Nishimura, Kakizaki et al. 2002).

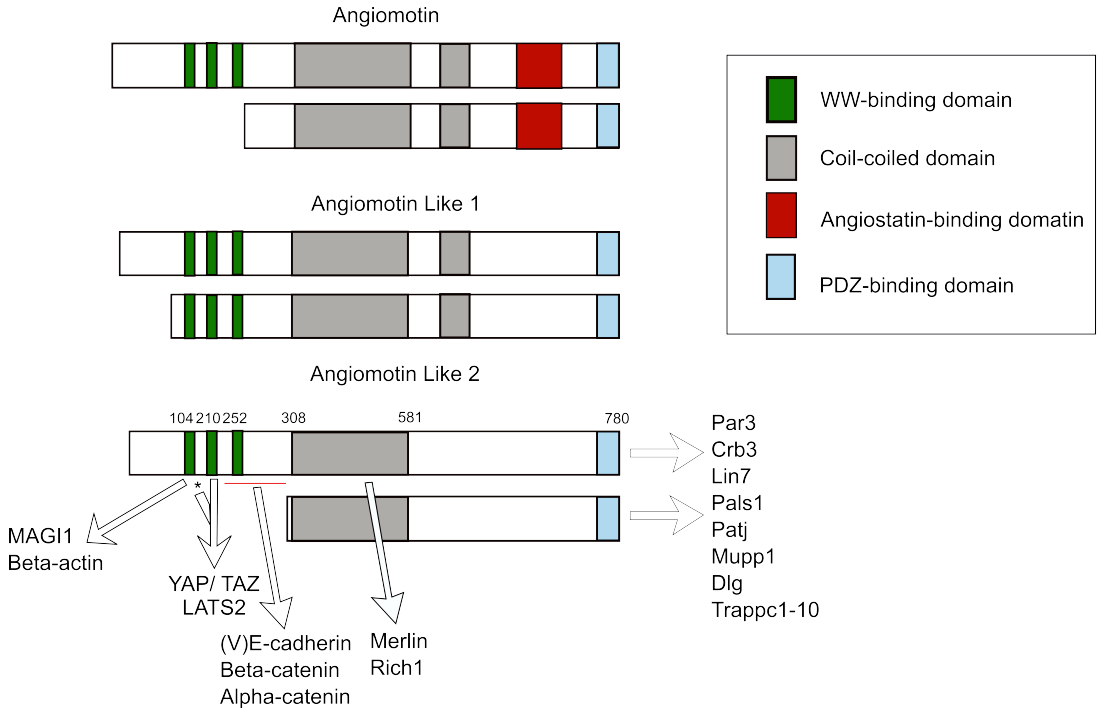

Figure 9. The Angiomotin protein family and interaction partners. The Angiomotin family consists of three members. Each family member exists as two different isoforms; Amot p130 and p80, AmotL1 p100 and p90, AmotL2 p100 and p60. AmotL2 interacts with regulators of the actin cytoskeleton, tissue growth, cell-cell junctions, apical-basal polarity and vesicle transport. MAGI1 and β-actin interacts with the first WW-binding domain, while YAP/ TAZ and LATS2 are associated to the second. In addition, the LATS2 interaction is strengthened by a phosphorylation (S159, *). Through its coil-coiled domain Angiomotin was shown to interact with Merlin and Rich1, regulators of Cdc42/ Rac1 GTPase activity. Moreover, in Paper II of this thesis, we were able to map the E-cadherin interaction to a domain of 87 amino acids (a.a. 220-307). Many proteins, in control of polarity, junctions and vesicle transport interact with the C-terminal PDZ-binding motif of AmotL2.

The role of the Angiomotins in angiogenic sprouting

Angiomotin was found to exist as two different isoforms driving entirely different cellular processes. While the longer isoform was involved in regulation of cell morphology and junction stability, the shorter isoform was responsible for the initially observed pro-migratory effects (Bratt, Birot et al. 2005) (Ernkvist, Aase et al. 2006). Both isoforms were shown to execute their functions through interactions with the actin cytoskeleton, in collaboration with different binding partners and with different spatial distributions (Wells, Fawcett et al. 2006) (Aase, Ernkvist et al. 2007) (Ernkvist, Luna Persson et al. 2009) (Yi, Troutman et al. 2011). Interestingly, endothelial expression of Angiomotin appeared specific to angiogenic- and remodeling vessels, and its depletion resulted in dilated cranial- and intersegmental vessels in mice and zebrafish embryos (Aase, Ernkvist et al. 2007). Targeting of Angiomotin by DNA vaccination resulted in decreased endothelial cell migration, angiogenesis and vessel permeability, and further reduced tumor growth when used in combination with a vaccine against EGFR2 (Her2)/ Neu (Arigoni, Barutello et al. 2012) (Holmgren, Ambrosino et al. 2006).

Just like its sibling, also Angiomotin Like-1 (AmotL1) presented with two different isoforms, with the shorter promoting more migratory cellular properties. In addition, both isoforms were shown to control tight junction formation, and appeared to be of

particular importance for the connection between the dorsal aorta and the sprouting intersegmental vessels in developing zebrafish embryos (Zheng, Vertuani et al. 2009).

The third member, Angiomotin Like-2 (AmotL2), was suggested to promote angiogenesis as a response to mitogenic FGF2 signaling, through activation of the MAPK-ERK pathway (Wang, Li et al. 2011). Interestingly, AmotL2 expression levels have been shown to be hypoxia regulated. Two different studies could demonstrate increased expression of AmotL2, already after 3h and 8h of hypoxia, respectively (Mondon, Mignot et al. 2005) (Liu, Laurell et al. 2007). In Paper IV we were able to tie the hypoxia-regulated feature to the shorter (p60) AmotL2 isoform. In addition, the Angiomotins were discovered to play important parts during morphogenic processes, also in non-endothelial tissues, for example epiboly and convergent extension during gastrulation (Huang, Lu et al. 2007) (Shimono and Behringer 2003).

The Angiomotins and the Hippo-signaling pathway

Recently, the Angiomotins were found to interact with members of the Hippo-signaling pathway, and were hence ascribed a novel role as regulators of contact inhibition of proliferation (Wang, Huang et al. 2011) (Zhao, Li et al. 2011) (Chan, Lim et al. 2011) (Paramasivam, Sarkeshik et al. 2011). The Hippo-signaling pathway controls cell growth, -proliferation and apoptosis, and is induced upon cell-cell contact formation. As already mentioned in previous sections, the Hippo-signaling pathway consists of MST- and LATS-kinases, in upstream control of the transcriptional co-activators YAP and TAZ. Phosphorylation of YAP/ TAZ by the kinases, prevents their nuclear translocation and hence dissociates them from their transcriptional targets. Several different routes of the Angiomotin-mediated negative control of YAP/ TAZ have been suggested. Most studies involve N-terminal Angiomotin domains, focusing the interest on the longer isoforms.

Firstly, the Motins can possibly directly interact with YAP/ TAZ, trapping them in the cytoplasm or at the cell junction and further promote their degradation. This interaction seems to be independent of LATS-mediated phosphorylation of YAP (Wang, Huang et al. 2011) (Chan, Lim et al. 2011) (Adler, Heller et al. 2013).

Secondly, the Angiomotins can, through interaction with upstream kinases, stimulate the ability of LATS to phosphorylate and hence inactivate YAP/ TAZ (Paramasivam, Sarkeshik et al. 2011). To complicate things even more, Angiomotins can probably also themselves be targets of LATS-phosphorylation, causing reinforcement of the LATS-interaction and disruption of the connection of Angiomotins to actin filaments (Dai, She et al. 2013) (Chan, Lim et al. 2013). The actin binding- and YAP-interaction sites seem to be mutually exclusive, since YAP overexpression totally abolishes the actin-binding capacity of the Angiomotins. Subsequently, modulations of actin cytoskeletal integrity proved to shift the binding preference of the Angiomotins to actin filaments or -YAP, respectively (Mana-Capelli, Paramasivam et al. 2014).

Last year another path for Angiomotin-regulated YAP localization and -activity was discovered, upon culturing of cells in low glucose environments. Such metabolic stress caused stabilization the Angiomotins through phosphorylation by the AMP-activated Protein Kinase (AMPK). The enhanced stability of Angiomotin resulted in phosphorylation- and inactivation of YAP (DeRan, Yang et al. 2014).

Additionally, the Angiomotins can affect the Hippo-signalling pathway and further YAP/ TAZ activity, indirectly through an interaction with Merlin. Merlin binds to the coil-coiled domain of the Angiomotins, and has been described as a regulator of contact inhibition of proliferation (Yi, Troutman et al. 2011). Merlin performs its actions by inhibiting the MAPK-pathway and receptor tyrosine kinases, as well as by affecting the Hippo signaling pathway (Yi, Troutman et al. 2011) (Hamaratoglu, Willecke et al. 2006).

In Drosophila, the Merlin ortholog acts in synergy with the adherens junction protein Expanded to phosphorylate Wts (mammalian LATS), and hence inactivate Yorkie (mammalian YAP) (Hamaratoglu, Willecke et al. 2006).

The Angiomotin-YAP interaction and their spatial distribution were lately found to regulate cell fate during early embryogenesis and blastocyst formation. In this system, Angiomotin appears to promote pluripotency of unpolarized cells of the inner cell mass (ICM). Establishment of apical-basal polarity changes the localization of Angiomotin from ubiquitous membrane expression, to expression restricted to the apical membrane domain. When expressed throughout the membrane, as is the case in unpolarized ICM cells, Angiomotin retains YAP in the cytoplasm. On the contrary, in polarized trophoectoderm cells where Angiomotin localization is restricted to the apical membrane, YAP is able to translocate into the nucleus. Both Hippo-pathway dependent- and independent mechanisms of the polarity-mediated YAP translocation have been suggested (Leung and Zernicka-Goetz 2013) (Hirate, Hirahara et al. 2013).

The association of the Angiomotins to cell-cell junctions and cadherins

As mentioned above, the Angiomotins have been linked to several different junction associated proteins (Pals1, Patj, Merlin etc.) (Ernkvist, Luna Persson et al. 2009, Yi, Troutman et al. 2011). Of special interest for this thesis is the link to the scaffold protein Membrane Associated Guanylate Kinase protein (MAGI-1) (Bratt, Birot et al. 2005, Patrie 2005). MAGI-1 was shown to associate to β-catenin and to promote adherens junction maturation and -strengthening through recruitment and activation of the small GTPase Rap1 (Dobrosotskaya and James 2000) (Sakurai, Fukuhara et al. 2006). In this thesis a connection between AmotL2 and the (V)E-cadherin/ catenin adherens junction protein complex was found. Furthermore, AmotL2 was proven essential for the association of MAGI-1 and β-actin to the cadherin/catenin complex. The AmotL2 interaction with the (V)E-cadherin/ catenin complex at the adherens junction promoted formation/ organization of contractile radial actin filaments, regulating epithelial- and endothelial cell morphology. Furthermore, we hypothesize these radial actin filaments to be of importance for intercellular communication and -organization (Hultin, Zheng et al. 2014). Interestingly, the shorter isoform of Angiomotin was recently found to associate to a mesenchymal cadherin (OB-cadherin). The interaction promoted migration in a prostate cancer cell line, providing a possible link of Angiomotins to the cadherin-switch commonly occurring during EMT and tumor dissemination (Ortiz, Lee et al. 2014).

Aims of the thesis

The general aims of the thesis were to study the role of Angiomotin Like 2 in vascular development, cell morphology and epithelial sheet formation as well as hypoxia-driven tumor progression.

Specific aims of the four projects:

Paper I
To analyze if and how AmotL2 is needed during vascular development, using mouse- and zebrafish models.

Paper II
To study whether AmotL2 functions also in epithelial tissues, further exploring its role in control of junction establishment and regulation of the actin cytoskeleton, and resulting consequences for cellular morphology and epithelial sheet formation.

Paper III
To investigate the recently discovered link between AmotL2 and the polarity protein Par3, and further how the interaction affects organization of the actin cytoskeleton and cellular morphology.

Paper IV
To explore the nature of the hypoxia responsive AmotL2 expression, and further how it correlates to, or even can be causally linked to, tumor progression.

Model systems

The zebrafish

The use of the zebrafish as an experimental model has become increasingly popular the last decades. The large egg clutch size facilitates accurate statistical calculations and the small body size allows the zebrafish to be maintained at high densities and consequently lower costs, compared to mammalian animal models. The initial development occurs rapidly during the first days of embryogenesis, enabling phenotypic analysis only after a few days. The zebrafish model is especially suited for imaging analysis techniques, as the embryos develop externally and can initially be kept transparent through prevention of pigmentation. In combination with increased transgenic model resources, the transparency facilitates live imaging of internal organs and processes. In the work of this thesis we used the zebrafish model for analysis of the vascular- as well as epidermal development. Since the early embryos are not dependent upon blood flow for oxygen and nutrient supply, it is possible to study also severe vascular phenotypes in living embryos. Additionally, reverse genetics have revealed genes controlling vascular development to be highly conserved, in between zebrafish and mammals (Lawson and Weinstein 2002). Although, many of the mammalian zebrafish orthologues exist on two different chromosomes due to whole genome duplication (Meyer and Schartl 1999).

Anti-sense oligonucleotides as well as gene coding constructs can easily be introduced into the zebrafish, through injection into the egg. Classical mammalian knockout techniques are commonly relying on homologous recombination. This technique has not been quite as successful in the zebrafish model, most likely due to the lack of proper embryonic stem cells. Instead, the most commonly used technique to induce gene silencing in zebrafish has been injection of anti-sense Morpholino (MO) oligonucleotides. Similar to siRNA methods, these constructs induce only a transient and incomplete knock down. The constructs are stable, and bind to RNA to interfere with either translation (through binding to the target start site) or splicing (through binding to splice site junctions). As the injection usually is performed at the one-cell-stage, the knockdown is global. Although, recently more advanced time- and space-restricted morpholinos have also been produced. Again, just like siRNAs, morpholinos are prone to induce off-target effects, for example neuronal apoptosis as a response to p53 upregulation. Therefore, stringent control experiments like usage of multiple morpholinos and rescue experiments where the MO is co-injected with mRNA encoding the target gene, are crucial. The last years several new methodologies for genetic deletion in the zebrafish model have been developed (zinc-finger nucleases, TALENs, CRISPR/ Cas9 etc.).

Moreover, in Paper I we take use of several different transgenic zebrafish models to analyze the early vascular development, cells of the vascular system and intracellular stuctures, respectively. In these transgenic models expression of a gene-of-interest is induced under a specified promoter, restricting overexpression of the protein to a specific cell population (where the promoter is active). For example in Paper I, we induce expression of the EGFP fluorochrome under the kdrl (VEGFR2) promoter and dsRed under the gata1 promoter, resulting in visualization of EGFP in endothelial cells and dsRed in the erythrocytes. Alternatively, we use the fli1 promoter to visualize the endothelial cells. To enable imaging of the actin cytoskeleton, we developed a transgenic model stemming from two different lines, using the GAL4/ UAS system. Firstly a tissue-specific promoter, in our case VE-cadherin, drives expression of the yeast transcription factor GAL4. In the other line, a construct encoding the gene-of-interest, as well as GAL4-

responsive Upstream Activating Sequences (UAS), is integrated. Crossing of the two lines results in binding of GAL4 to the UAS, and consequent transcription of the gene-of-interest, solely in cells where the VE-cadherin promoter is active (Lawson and Wolfe 2011). In particular, we were interested in inducing expression of the actin-binding peptide LifeAct, coupled to EGFP. We also took use of another GAL4/ UAS zebrafish line, where both mRFP and VE-cadherin-EGFP are expressed under the fli1 promoter. In our analysis of aortic endothelial cell morphology, we used another transgenic system, based on the Tol2 Transposase. In this method, transposase mRNA is co-injected with a construct encoding the gene-of-interest, flanked by Tol2-elements. The Tol2 transposase acts to cut the genomic DNA, to allow for integration of the construct and further expression of the gene its encoding (Fisher, Grice et al. 2006).

The loxP/Cdh5(PAC)-CreERT2 mouse

As mentioned in the previous paragraph, classic transgenic models are usually based on homologous recombination. This is true also for the Cre-lox system, where the Cre enzyme recombines the loxP sites, resulting in the deletion of the gene these sites are spanning. In this specific model, the Cre-recombinase is linked to the estrogen receptor and hence activated upon injection of the receptor ligand tamoxifen. Cre-ERT2 is expressed under the Cdh5(PAC) (VE-cadherin) promoter, restricting the gene deletion to endothelial cells (Sorensen, Adams et al. 2009) (Pitulescu, Schmidt et al. 2010). Furthermore, to evaluate the efficacy of the Cre-recombinase, we crossed our AmotL2$^{lox/lox}$/Cdh5(PAC)-CreERT2 mice to ROSA26-EYFP reporter mice. In these mice, a transcriptional termination site flanked by loxP sites precedes the EYFP gene. Furthermore, Cre-recombinase targeting to the loxP-sites removes the termination site, and allows expression of EYFP. Consequently, EYFP expression can be correlated to Cre-activity and further knock out efficiency of our gene-of-interest (Srinivas, Watanabe et al. 2001).

Results and discussion

Paper I

AmotL2 links VE-cadherin to contractile actin fibres necessary for aortic lumen expansion

AmotL2 is required for dorsal aorta development

In this paper we aimed to investigate the function of Angiomotin Like 2 (AmotL2) during embryonic vascular development. There are two zebrafish orthologues to the mammalian *AmotL2*, localized to chromosome 6 (*amotl2a*) and -2 (*amotl2b*) respectively. Loss of AmotL2a was previously shown to result in gastrulation defects in the zebrafish model (Huang, Lu et al. 2007). To be able to analyse the effect on the vascular system we hence reduced the concentration of the anti-sense morpholinos, to allow for development post gastrulation. We individually targeted *amotl2a* and *–b*, in transgenic embryos were endothelial cells and erythrocytes can be visualized by the two fluorochromes EGFP and dsRed, respectively. Targeting of both genes resulted in loss of circulation, accompanied by development of a pericardial oedema. In contrast to vasculature of control embryos, no circulating erythrocytes could be detected within the trunk vasculature of *amotl2a* or *–b* embryos, even though the heart was beating. The erythrocytes where instead accumulated in association to the pericardial oedema. A combination of *amotl2a* and *–b* morpholinos somewhat enhanced the severity of the phenotype, compared to individual targeting of the two paralogues. When the embryos were analysed in more detail with the prospect to identify the cause of the circulatory defect, it became evident that the dorsal aorta (DA) suffered from constrictions, while the pericardial vein (PCV) appeared unaffected.

Why is the amotl2 MO vascular phenotype restricted to the dorsal aorta?

Arteries and veins are known to experience different magnitudes of shear stress, due to differences in blood pressure. Since arteries constantly are exposed to higher pressure, it is possible that also arterial lumen formation is more dependent upon blood flow. Hence, we decided to investigate whether lack of circulation per se could induce arterial constrictions. To this end, we targeted the cardiac Troponin Type 2 (*tnnt2*), a depletion known to result in cardiac arrest and consequent absence of circulation in zebrafish embryos (Sehnert, Huq et al. 2002). Since the first days of embryonic development in zebrafish is not dependent upon circulation for uptake of oxygen and nutrients, this model would allow us to independently study the effects of loss of circulation. Interestingly, the aortic lumens of the *tnnt2* morphants were observed to be slightly smaller in comparison to lumens of control embryos, but no constrictions were detected. This argues for a flow-independent mechanism of vascular lumen expansion, at least during the initial vascular development in the zebrafish model.

Instead the phenotypical differences between the arteries and veins can stem from different modes of lumen formation. The endothelial cells contributing to arteries and veins respectively, have been proven to differentiate already prior to midline migration. The different cells express different molecular signatures and are clearly distinct subpopulations of endothelial cells (Lawson and Weinstein 2002) (Kohli, Schumacher et al. 2013). *amotL2* itself seem to be homogenously expressed throughout the vascular plexus (Huang, Lu et al. 2007). Nevertheless, its function might be dependent upon

interaction with partners expressed exclusively in the arterial compartment. Furthermore, the actual mechanism of lumen formation might differ between arteries and veins (Herbert, Huisken et al. 2009) (Helker, Schuermann et al. 2013). Previously, an artery-specific phenotype was observed in mouse aortae depleted of β1-integrin. The function of β1-integrin in aortic lumen formation was shown to be dependent on an interaction with the apical polarity protein Par3 (Zovein, Luque et al. 2010). Interestingly, we have shown Par3 to associate to AmotL2 (Paper IV), suggesting the two proteins to be part of a common signaling pathway. This idea will be elaborated in more detail in the discussion of Paper III.

In line with the findings of Wang et al, we did observe a slight delay in intersegmental vessel (ISV) sprouting in the *amotl2* morphants. However we were not able to detect any sustained effects after 48 hours post fertilization (hpf).

Actomyosin dependent cell shape changes controls aortic development and possibly - sustainment

Strict control of cell shape is one of the requirements for proper tubulogenesis (Lubarsky and Krasnow 2003) (Pilot and Lecuit 2005). To look into the cause of the arterial lumen defects, we therefore decided to analyse the aortic endothelial cell morphology. By mosaic endothelial expression of a construct encoding the fluorochrome mCherry, we were able to analyse the morphology of individual cells *in vivo* in living zebrafish embryos. The endothelial cells of the *amotl2* morphants possessed a more rounded morphology, compared to the thin and elongated appearance of the endothelial cells of control embryos. The altered cell shape was not due to loss of circulation, as endothelial cells of *tnnt2* MO embryos showed an elongated phenotype similar to ECs of control embryos.

As described and discussed throughout the introduction of this thesis, cell shape is determined through tight interactions of several cellular processes including apical-basal polarity, cell-cell junctions and the actin cytoskeleton. To explore possible defects within any of these processes, we performed immunofluorescence staining of fixed control and *amotl2* MO zebrafish embryos. Decreasing the AmotL2 levels did not affect establishment of apical basal polarity, as judged by the localization of the apical glycoprotein podocalyxin and the basal lamina component laminin. Adherens (VE-cadherin)- and tight junction (Zonula Occludens-1 (ZO-1)) proteins still localized to cell-cell contacts, but their expression pattern appeared slightly altered in the *amotl2* MO embryos. This might be due to the altered cellular morphology, or alternatively, reflect changes in dynamics- and stability of the junctions. The dynamics of cell-cell junctions are reported to be highly dependent on the association to supporting actin structures (Verma, Han et al. 2012).

To investigate whether defects in the actin cytoskeleton could be responsible for the altered endothelial cell morphology, we used a transgenic zebrafish model were the F-actin binding peptide LifeAct was expressed in the endothelial cells and linked to EGFP. In aortic endothelial cells of control embryos, LifeAct labelled both circumferential (cortical) actin and radial actin filaments. Up on *amotl2* depletion, the cortical actin structures were still present, while the radial actin filaments were almost completely abrogated. The role of the actin cytoskeleton in aortic development was further strengthened by treatment of the zebrafish embryos with different actin/ actomyosin-targeting drugs. Blebbistatin, Cytochalasin D and the ROCK-inhibitor Y-27632, all resulted in aortic constrictions and consequent loss of circulation. As the myosin II inhibitor blebbistatin was previously shown to impede heart contractions in zebrafish

embryos, we used lower concentrations unable to affect the heartbeat (Jou, Spitzer et al. 2010). Interestingly, blebbistatin treatment did not only inhibit onset of circulation, but also rapidly terminated it when treatment was performed at 48 hpf, well after onset of circulation but before recruitment of vascular smooth muscle cells. This finding, argues for a need of endothelial actomyosin forces, not only throughout development but also for maintenance of arterial vessels. The importance of proper regulation of the actin cytoskeleton during vascular development has been proposed before, for example in studies of the Rap1 effector Rasip1 in the developing mouse aortae (Xu, Sacharidou et al. 2011).

Comparison of the AmotL2 function in the zebrafish and mouse model

The zebrafish model is commonly used for studies of embryonic vascular development. The morpholino knock down technique allows for rapid, but transient, gene silencing and phenotype analysis in the zebrafish embryo. However, even numerous control experiments are not able to totally control for morpholino-induced non-specific off-target effects. To assure the validity of the observed *amotl2* MO phenotype, and further analyse the importance of AmotL2 in mammalian vascular development, we developed an *Amotl2* knock out mouse. We used the Cre-lox system to induce deletion of *Amotl2* under the VE-cadherin promoter. Activity of the VE-cadherin promoter has been observed in the yolk sac as early as E7.5, still full penetrance is not accomplished until E14.5. As the lumens of the paired dorsal aortae start to expand around E8, this model would result in a slightly incomplete knock out, at the time of aortic lumen development (Alva, Zovein et al. 2006) (Strilic, Kucera et al. 2009). However, at E9.5 the *AmotL2* knock out embryos presented with a convincing phenotype. Constrictions were found along the length of the dorsal aortae, strongly mimicking what we previously observed in the zebrafish model. On the contrary, we were not able to demonstrate any defect on neither embryonic- nor postnatal angiogenesis.

Furthermore, since single allele targeting in the zebrafish model was sufficient to create a phenotype, we suspected that also an incomplete deletion of *AmotL2* in the mouse would do so. In line with this finding, not only homozygous, but also heterozygous mouse embryos presented with aortic constrictions. Off interest, constrictions were not only found along the length of the dorsal aortae, but also in branchial arteries in connection with the heart, possibly representing areas of high blood pressure. It is thus tempting to speculate that the AmotL2 expression level and function is determined by the magnitude of blood pressure/ shear stress. If so, this opens up for interesting possible implications, within the field of atherosclerotic plaque development.

Can AmotL2 be the missing link, connecting cadherins to the actin cytoskeleton?

In this study we were able to link the AmotL2-mediated actin cytoskeleton regulation to interactions with the VE-cadherin/ catenin adherens junction protein complex. Already earlier studies could show an indirect association of AmotL2 to the cadherins through interaction with MAGI-1 (Patrie 2005) (Dobrosotskaya and James 2000). We were here able to take this idea further by showing the presence of AmotL2, to be crucial for the connection of MAGI-1 and β-actin to the VE-cadherin/ catenin protein complex. This is of interest, as recent studies have questioned the conventional idea of α-catenin as a linker between cadherins and the actin cytoskeleton (Yamada, Pokutta et al. 2005). Our findings, argue for a role for AmotL2 in linkage of the cytoskeleton to the cadherins/ catenins, and further for cell-cell contact induced generation of junction associated actin filaments. Furthermore, we could show AmotL2 to specifically control the structure/ organization of the radial actin filaments in contrast to the cortical actin, which seemed

fairly intact even in the absence of AmotL2. Possibly, AmotL2 could act in conjunction with other actin-associated proteins (eg. EPLIN), to control different actin filament subtypes and regulate cellular morphology (Taguchi, Ishiuchi et al. 2011).

Does AmotL2 function as a mechanosensor?

The radial junction-associated actin filaments appear to be contractile, as AmotL2 depletion and consequent loss of the radial filaments resulted in decreased ability of the cells to generate force.

We hypothesize the AmotL2-dependent radial fibers to reach over the length of individual cells and hence connect neighbouring cells. If as suspected, the targeted actin filaments posses contractile properties and further link adjacent cells, it would argue for a role for AmotL2 in mechanical force sensing. Possibly AmotL2 is part of a population of mechanosensory actin-associated proteins, showing distinct specificity for different types of actin filaments- and grade of junctional maturation (Taguchi, Ishiuchi et al. 2011) (Huveneers, Oldenburg et al. 2012).

Paper II

The E-cadherin/AmotL2 complex controls hexagonal packing of epithelial cells

AmotL2 is ubiquitously expressed in epithelial tissues

In this paper we aimed to explore whether AmotL2 controls actin cytoskeleton organization and further cell morphology also in epithelial cells. To analyze and compare tissue level expression of AmotL2 we used the GeneSapiens database. The database summarizes data from normal- and pathological human tissue samples, as well as cell lines, stemming from publicly available microarray experiments. We found *AMOTL2* mRNA to be expressed in most tissues, with the exceptions of lymphoid-, blood- and bone marrow lineages. In addition, *AmotL2* expression was previously published to be especially high in heart, liver, kidney, lung and skin. In line with previous studies, we found the AmotL2 protein to be localized to cell-cell junctions of epithelial cell lines and zebrafish epidermis (Patrie 2005). To enable studies of AmotL2 in epithelial tissues, we created *AMOTL2* knock down cells by usage of lentiviral vectors. Similarly to our previous findings (Paper I) these cells were still able to polarize in an apical-basal fashion, and their tight junctions appeared intact. An E-cadherin signal could still be detected at cell-cell junctions, but it seemed more diffuse and the protein was more often found intracellular.

Does AmotL2 affect cell-cell junction formation and/ or -dynamics?

To examine junction formation and -integrity we performed a Trans Epithelial Resistance (TER) assay, after exposing our cells to a calcium switch. Since cadherin-based junctions are calcium dependent they can be disassembled upon calcium removal, and subsequently re-established when calcium levels are restored. As the junction matures the cell-cell connection is successively tightened, represented by a peak in TER. *AMOTL2* shRNA cells reached the TER-peak at the same time as corresponding control cells, but never managed to obtain the same magnitude of junctional integrity. These observations are similar to findings from experiments where the apical tight junction protein Par3 was depleted and actin cytoskeletal organization disrupted (Chen and Macara 2005).

Cell shape and tube formation – what is the connection?

Upon assembly of epithelial cells into a multicellular sheet, the cells usually obtain a hexagonal shape to optimize packing and minimize surface energy. The polygonal distribution is preserved in between tissues and species (Gibson and Gibson 2009). Junctional length and further cell geometry are the consequences of junctional protein activity, as well as actomyosin contraction (Lecuit and Lenne 2007). In *AMOTL2* shRNA cells, the constant polygonal distribution was lost. Instead, the cells more frequently obtained rectangular and triangular shapes. In addition, the altered morphology of the *AMOTL2* shRNA cells was clearly demonstrated by a cell surface area around six times larger than the area of control cells. We were not able to detect any defects in the microtubule- or intermediate filament networks. On the contrary, the actin cytoskeleton was disorganized, and especially radial actin filaments appeared totally disrupted/ depleted. As also treatment of control cells with the myosin II inhibitor blebbistatin resulted in cells with enlarged area and altered polygonal distribution, we argue for loss of contractile radial actomyosin filaments being the cause of the observed *AMOTL2* shRNA phenotype.

Eventhough *AMOTL2* shRNA did not affect apical-basal polarity in cells grown as a monolayer, those cells were not able to organize into polarized three-dimensional lumenized structures. The differences in the results obtained from monolayer- compared to 3D settings, could possibly be explained by the need for actomyosin dependent cell shape changes and force generation during lumen expansion (Strilic, Kucera et al. 2009). Absence of such intercellular coordination and force production might result in collapse of the cyst, causing a disorganized and unpolarized structure. In addition experiments where cell shape was confined through culturing of cells on area-restrictive molds, suggest cell spreading (and hence enlarged cell surface area), to interfere with lumen formation (Rodriguez-Fraticelli and Martin-Belmonte 2013).

Radial actin filament versus cortical actin – who does what?

Loss of the radial actin filaments also affected the cellular resistance to pulling forces. Cells were cultured on to a silicone membrane, and further exposed to tearing forces. Membrane stretching resulted in disruption of control cell-cell contacts, while cell sheets consisting of *AMOTL2* shRNA- or blebbistatin treated cells, remained intact throughout the experiment. Radial actin filaments have previously been shown to weaken junctions through their contractile features (Millan, Cain et al. 2010). Perhaps, the lack of intracellular pulling forces from the radial filaments and further the inability to sense forces from their neighbors, makes the *AMOTL2* shRNA cell sheet stay intact even under tearing forces.

How does AmotL2 interact with the Hippo-signaling pathway and YAP?

Several papers have been published the last years presenting evidence for a role of AmotL2 in control of Hippo-signaling and YAP-activation (Wang, Huang et al. 2011) (Paramasivam, Sarkeshik et al. 2011). Consistently, data from the GeneSapiens database also demonstrated a clear correlation between expression of AmotL2 and members of the Hippo-signaling pathway. To investigate the relationship in more detail, we analyzed the localization of the transcriptional co-activator YAP in control- and *AMOTL2* shRNA cells. *AMOTL2* shRNA cells grew slightly slower, but reached contact inhibition of proliferation (CIP) at a similar time-point compared to controls. As the Hippo pathway are in control of CIP, this argues against a role for AmotL2 in control of YAP localization and –activation. Consequently when cell culturing was continued post cell-cell contact formation, YAP was eventually translocated to the cytoplasm in both control- and

AMOTL2 shRNA cells. Hence, the nuclear-to-cytoplasm transport mechanism remains intact even in the absence of AmotL2.

Previously, an alternative mechanism of YAP inactivation, was suggested to act indirectly through effects on the actin cytoskeleton. Disruption of the actin cytoskeleton resulted in Angiomotin-mediated inhibition of YAP. Furthermore, the YAP- and actin binding capacities of the Angiomotins, were shown to be mutually exclusive. Interfering with the actin cytoskeleton/ actin-binding capacity of the Angiomotins, enhanced the interaction with YAP. In paper II we present an alternative mechanism, as YAP translocation to the cytoplasm remained functional also in the absence of AmotL2 (Mana-Capelli, Paramasivam et al. 2014).

In addition, cell shape has been shown to be a determinant of YAP localization, possibly through effects on the actin cytoskeleton (Dupont, Morsut et al. 2011) (Aragona, Panciera et al. 2013). We were able to repeat these results by controlling the areas of single epithelial cells. As single cells do not form any cell-cell junctions, neither would the AmotL2-dependent radial actin filaments be of any importance under these growth conditions. Consequently, YAP translocation to the nucleus was dependent on cell area, but independent of AmotL2 expression status.

How does AmotL2 interact with E-cadherin and the actin cytoskeleton, respectively?

In Paper I we found AmotL2 to associate to the VE-cadherin/catenin adherens junction protein complex. This finding inspired us to look for a similar interaction within epithelial cells. Indeed, we found AmotL2 to interact with E-cadherin, as well as β-catenin, α-catenin, MAGI-1 and β-actin. Similarly to our findings from the endothelial cells (Paper I), we could map the interaction to MAGI-1, as well as β-actin, to a LPTY motif (Y107) in the N-terminal domain of AmotL2. This actin-binding site differs from the one previously identified by Mana-Capelli et al.. Possibly, both binding sites act in conjunction to achieve association to the actin filaments. As mutating the MAGI-1 binding site only interfered with the AmotL2 connection to β-actin, but not to the E-cadherin/ catenin complex, we conclude that E-cadherin interacts with AmotL2 through a distinct site. We were able to identify a N-terminal 87 a.a. domain as responsible for the E-cadherin binding, yet the exact site of interaction is unknown. Angiomotin binds Merlin, which was in turn previously shown to interact with α-catenin (Gladden, Hebert et al. 2010). Hence, one possibility would be an indirect association of the Angiomotins to E-cadherin, through Merlin and α-catenin. However, E-cadherin was shown to interact with Angiomotin independently of its β-catenin (and further α-catenin-) binding domain (Hirate, Hirahara et al. 2013). Additionally, the Merlin interacting site of Angiomotin (coil-coiled domain) is not compatible with the domain we identified as important for the association to E-cadherin (220-307 a.a.). The exact constellation of the protein complex still remains to be discovered.

Paper III

AmotL2 integrates polarity and junctional cues to modulate cell shape

As we recently found AmotL2 to interact with the polarity protein Par3 through the C-terminal PDZ-binding motif (Paper IV), we here aimed to investigate this link further, especially focusing on effects within actin cytoskeleton organization and cell morphology.

Do AmotL2 and Par3 cooperate to control embryonic dorsal aorta development in the zebrafish?

Par3 was previously ascribed a role during aortic tubulogenesis (Zovein, Luque et al. 2010). Hence, our recent findings regarding AmotL2 in aortic development (Paper I) in combination with the AmotL2-Par3 interaction studies (Paper IV), prompted us to study a possible joint mechanism of action during aortic expansion. A zebrafish orthologue of the mammalian Par3 was previously discovered and depleted using anti-sense morpholinos, resulting in severe brain- and eye defects (Wei, Cheng et al. 2004). Therefore, to avoid serious developmental defects and allow for analysis of the vascular development, we used reduced morpholino concentrations. At 48hpf, the *par3* MO embryos presented with pericardial- as well as brain oedema. When analyzed in a double transgenic background, we were able to detect an accumulation of the dsRed positive erythrocytes in association to the pericardial oedema, instead of circulating within the EGFP-positive vascular network. When examined in more detail it became clear that *par3*-, similarly to *amotL2* morphants, only suffered from constrictions within the dorsal aorta. In contrast, the pericardial vein remained lumenized. The *amotL2* MO- and *par3* MO phenotypes strongly mimicked each other and furthermore the *par3* MO corroborated the findings from the mouse aorta (Zovein, Luque et al. 2010). The importance of the AmotL2-Par3 interaction during aortic development was further strengthened by the need for the Par3-interacting PDZ-binding motif of AmotL2, to allow for rescue of the circulatory phenotype.

In addition to a role in vascular development, we found AmotL2 to control cell shape through actin cytoskeleton organization in the zebrafish skin (Paper II). Consequently, also silencing of Par3 affected morphology of the epithelial skin cells, as determined by cell area and polygonal distribution. Similarly to the phenotype of *amotL2* morphants, the morphological alteration of the *par3* MO epidermal cells appeared to be caused by loss of the radial actin filaments. Both epithelial cells of the zebrafish skin, and endothelial cells *in vitro*, presented with disorganized and disrupted actin cytoskeleton architecture in the absence of Par3.

Is AmotL2 function dependent upon Par3?

To further analyze the relationship between AmotL2 and Par3, we individually targeted both genes using siRNA in mouse endothelial cells *in vitro*. Interestingly, we found junctional localization of AmotL2 to be dependent on Par3. When Par3 was depleted, AmotL2 did not succeed to transport to the junction, but instead accumulated in cytoplasmic vesicles. On the contrary, AmotL2 levels did not affect the localization of Par3. Furthermore, Par3 silencing did not affect total AmotL2 levels, arguing for a regulation solely of the spatial distribution. Similarly, mutant constructs of Angiomotin unable to interact with and localize to F-actin structures, were previously observed to localize to intracellular vesicles (Mana-Capelli, Paramasivam et al. 2014). As Par3 was previously implicated in vesicle trafficking (Balklava, Pant et al. 2007), we continued to investigate whether Par3 could promote transport of AmotL2 to the cell-cell junction. To this end, we co-transfected bovine capillary endothelial cells with constructs encoding both proteins tagged to different fluorochromes, enabling live cell imaging of the protein dynamics. Interestingly, we found signals from both fluorochromes co-localizing to cytoplasmic vesicles, transporting to the cell-cell junction. We hypothesize, Par3 to control junctional targeting of AmotL2.

Is Par3 needed for association of AmotL2 to the (V)E-cadherin/catenin adherens junction protein complex?

Upon silencing of Par3 by siRNA, AmotL2 failed to be transported to the cell-cell junctions. In contrast, β-catenin localization appeared unaffected. We hence hypothesized the Par3-mediated transport of AmotL2 to be crucial for the ability of AmotL2 to associate to β-catenin and further (V)E-cadherin. To evaluate this hypothesis, we depleted immortal human keratinocytes of Par3, and analyzed the association of AmotL2 to members of the E-cadherin/ catenin protein complex. Indeed, loss of Par3 completely abrogated the interaction of AmotL2 to both E-cadherin, β-catenin and furthermore MAGI-1 and β-actin. This strongly suggests a role for Par3 in transporting AmotL2 to the adherens junction, where AmotL2 by interaction with the (V)E-cadherin/ catenin protein complex acts to organize radial actin filaments associated to the junctional complex.

Par3 – part of two separate protein complexes, in control of two distinct cellular processes?

Traditionally, Par3 was described as a protein in control of apical domain formation, as part of a complex with aPKC and Par6. Following initiation of the apical membrane specification, Par3 has been shown to be restricted to apical tight junctions (Tepass 2012). A few years ago, Iden et al, described a novel protein complex consisting of Par3 and the adherens junction protein VE-cadherin. In this study Par3 could associate specifically to VE-cadherin, but not to E- or N-cadherin (Iden, Rehder et al. 2006). In contrast, Wei et al reported association of Par3 to β-catenin and DE-cadherin of drosophila epithelial cells (Wei, Escudero et al. 2005). Interestingly, further studies demonstrated different E-cadherin pools to possess different dynamic properties, and Par3 to specifically associate to the more mobile E-cadherin fraction (Bulgakova, Grigoriev et al. 2013). In Paper III, we report a novel role for Par3 as a transporter of AmotL2, facilitating its association to the (V)E-cadherin/ catenin protein complex. Depletion of either AmotL2 or Par3 appears to preferentially affect radial actin filaments. Perhaps this novel Par3/ AmotL2/ (V)E-cadherin protein complex specifically associates to radial actin filaments of immature junctions and not to circumferential cortical actin structures.

Integration of polarity, junctions and the actin cytoskeleton

As Par3 was suggested to function downstream of β1-integrin signaling during aortic tube formation, we speculate integrin-activation to underlie the Par3-mediated transport of AmotL2 to the adherens junction. In turn the activation of β1-integrin might be stimulated by mechanical signals from the ECM. Traditionally, integrin activation has been linked to induction of focal adhesion associated actin filaments. Perhaps, by inducing the transport of Par3 and AmotL2 to the adherens junctions and further stimulating formation/ organization of junction associated actin filaments, the cell assures preservation of the balance between different actin cytoskeletal subtypes/ structures. Stimulation of contractile radial junction-associated actin filaments, could possibly be a way for the cell to counteract and balance pulling forces from the ECM, to maintain homeostasis of an epithelial sheet.

Paper IV

AmotL2 disrupts apical-basal polarity and promotes tumour invasion

AmotL2 expression was previously shown to be induced during hypoxic conditions (Mondon, Mignot et al. 2005) (Liu, Laurell et al. 2007). This project aimed to analyze this further, and in addition study possible implications for tumor progression.

How can hypoxia induce AmotL2 expression?

When evaluating breast- and colon tissue from tumor and adjacent normal areas, it became evident that AmotL2 expression was restricted to depolarized (carcinoma in situ) and invasive (invasive front of colon tumor) areas. The expression localized to areas of low oxygen concentration, as determined by carbonic anhydrase IX (CAIX) and glucose transporter I (GLUTI) staining. Interestingly, the AmotL2 signal stemmed from intracellular large vesicles, in contrast to the usual junctional localization (Paper I, II and III). AmotL2 exists as at least two different isoforms, with the shorter lacking the N-terminal glutamine-rich domain. Since vesicular localization was previously shown to be characteristic of the shorter p80 isoform of Angiomotin (Heller, Adu-Gyamfi et al. 2010), we decided to investigate whether AmotL2 expression in the tumor samples could be linked to the shorter isoform. Indeed, staining of the tissue samples with a N-terminal binding antibody was negative.

To specifically connect the shorter isoform to the hypoxia regulated feature of AmotL2, we exposed cells to 0.3% hypoxia for 8 hours, and analyzed mRNA expression of the two *AMOTL2* isoforms. Consequently, mainly the mRNA levels of the shorter (p60) isoform were enhanced. The induction was independent of the well-known hypoxia regulated factors HIF-1α and HIF-2α, as their silencing with siRNA did not prevent the enhancement of AmotL2 expression levels. Instead we were able to relate the induction of AmotL2 p60 expression to increased activity of the AP-1 transcription factor component c-Fos. c-Fos expression is known to be rapidly and transiently induced upon different stress stimuli. For example, c-Fos expression and further AP-1 DNA-binding activity were enhanced in ischemic areas of rat brain *in vivo* and after exposure of HeLa tumor cells to hypoxia *in vitro* (An, Lin et al. 1993) (Rupec and Baeuerle 1995). Of interest, c-Fos overexpression has been linked to epithelial cell depolarization and EMT (Reichmann, Schwarz et al. 1992). Perhaps the effect of c-Fos on apical-basal polarity is mediated through induction of AmotL2 p60 expression.

What type of vesicles is AmotL2 localizing to?

AmotL2 localization to the vesicular structures could be linked to the c-terminal PDZ-binding motif, as deletion of this site caused AmotL2 to instead be diffusely expressed throughout the cytoplasm. The PDZ-binding motif is common for both the longer (p100) and the shorter (p60) isoforms, still only AmotL2 p60 expression induced the large vesicles. Perhaps, the N-terminal association to E-cadherin and the actin cytoskeleton "traps" the longer AmotL2 p100 isoform in association with the cell cortex. Accordingly, Mana-Capelli et al., showed Angiomotin mutant constructs unable to interact with actin, to be localized to cytoplasmic vesicular structures (Mana-Capelli, Paramasivam et al. 2014). Heller et al., also suggested the shorter Angiomotin isoform, lacking the N-terminal actin interacting domain, to localize to endosomal vesicles (Heller, Adu-Gyamfi et al. 2010). In contrast, we were not able to detect any co-localization of AmotL2 to endosomal markers. Furthermore, the AmotL2 p60 positive vesicles were negative also for markers of the Golgi network, lysosomes and autophagosomes.

By using peptide pull-down followed by mass spectrometry, we were able to identify proteins interacting with the PDZ-binding motif. Previously detected interactions with the polarity proteins Patj, Pals1 and Par3 were reproduced (Wells, Fawcett et al. 2006). Consequently, expression of these proteins as well as the apical polarity protein Crb3, were detected at the AmotL2 p60 positive vesicles. Interestingly, we also identified proteins of the Transport Protein Particle (TRAPPII) complex, as AmotL2 binding partners. In order to evaluate whether the AmotL2 p60-induced vesicles and the consequent cytoplasmic capture of polarity proteins, could be due to interference with the TRAPPII- mediated vesicular transport, we depleted the TRAPPII component Trappc9 in mouse mammary gland epithelial cells. Trappc9 depletion caused Par3 re-localization from apical junctions to vesicles, similar to what we observed upon AmotL2 p60 overexpression. The TRAPPII protein complex has previously been implicated in control of cell polarity in the Arabidopsis plant, where depletion of the orthologues of Trappc9 and -10 resulted in defective targeted transport to the apical membrane (Qi, Kaneda et al. 2011). We concluded that AmotL2 p60 traps apical polarity proteins in cytoplasmic large vesicles, through interaction- and interference with the TRAPPII vesicle-transport pathway.

Does AmotL2 p60 promote tumor progression and an invasive phenotype?

Hypoxia has been shown to be inversely correlated to tumor cell polarity and to be associated with aggressive- and invasive tumor properties (Hockel, Schlenger et al. 1996, Yang, Wu et al. 2008). As AmotL2 p60 was shown to be induced in hypoxic tumor areas and to interact- and interfere with the localization of several polarity proteins, we hypothesized that it could indeed promote-, and not simply be a consequence of depolarization of epithelial structures. Previously repression of the polarity proteins Par3 and Crb3 were shown to promote tumorigenesis of breast and kidney cells (Karp, Tan et al. 2008, Xue, Krishnamurthy et al. 2013). Indeed, AmotL2 p60 overexpression resulted in depolarized cells and decreased junction integrity, similar to previously observed findings in epithelial cells depleted of Par3. Furthermore, AmotL2 p60 expression interfered with the cyst- and lumen-forming capacity of epithelial cells cultured in matrigel matrix, but did not promote invasion until addition of further stimuli. These findings strongly mimicked the previously observed effects of Par3 deletion on tumor progression and -invasion (Xue, Krishnamurthy et al. 2013).

Not only polarity proteins, but also the HGF-receptor c-MET re-localized as a response to AmotL2 p60 overexpression. Furthermore, AmotL2 p60 expressing cells to a higher extent responded to, and caused invasion into surrounding matrigel matrix upon HGF-stimulation. The transcription of C-MET has been shown to, just like AmotL2 p60 expression, be induced by hypoxia. Moreover, c-MET expression as well as HGF-signaling is commonly enhanced in tumors, specifically in areas of poor oxygenation. Furthermore, c-MET-HGF signaling in tumors can be correlated to EMT, invasive potential and poor outcome for the patient (Pennacchietti, Michieli et al. 2003) (Boccaccio and Comoglio 2006). As hypoxia and HGF act in synergy to promote tumor cell invasion (Pennacchietti, Michieli et al. 2003), it is tempting to speculate that the hypoxia-induced effect is at least partly mediated through increased expression of AmotL2 p60. Perhaps the AmotL2 p60-mediated receptor re-localization results in increased sensitivity to HGF cues.

The AmotL2 p60 effects on apical-basal polarity and metastatic potential could be reverted, as shown by depletion of endogenous AmotL2 p60 in the highly metastatic mouse mammary cell line 66c14. Following siRNA-mediated AmotL2 depletion, 66c14 cells were able to polarize and further form 3D cysts when cultured in matrigel matrix.

AmotL2 p60 depletion further decreased tumorigenicity following injection into a mouse model. This is in line with results from studies of c-Fos, where overexpression caused reversible loss of polarity (Reichmann, Schwarz et al. 1992) and -c-MET where inhibition of the receptor prevented hypoxia-induced invasion (Pennacchietti, Michieli et al. 2003).

AmotL2 p60 as a prognostic factor or drug target?

Several drugs targeting the c-MET tyrosine kinase receptor have been developed, and are currently being investigated for potential use in anti-tumor therapy (Comoglio, Giordano et al. 2008, Zhu, Kong et al. 2014). Since AmotL2 p60 potentiate c-MET-HGF signalling, it could also be of interest as a target for cancer therapy. As AmotL2 p60 does not appear to be expressed in normal quiescent adult tissue, it could possibly be a suitable target for treatment of aggressive and invasive tumors. In our study, AmotL2 expression strongly correlated to the clinical outcome in colon cancer. Hence it could additionally be used as a prognostic factor, perhaps identifying the patients who would benefit from treatment with c-MET inhibitors.

Most likely AmotL2 p60 acts in conjunction with other factors (Twist, Snail etc.) during transformation of epithelial cells and adaptation of migratory features. Perhaps, using a combinatory treatment simultaneously targeting several factors would be the solution to prevent hypoxic tumors from spreading and metastasizing.

Concluding Remarks and Future Perspectives

In the papers of this thesis we have identified a mechanism explaining how adjacent cells are linked through connection of transmembrane cadherins to cytoplasmic contractile actomyosin networks. Further we show these cadherin-associated actin filaments to be important for transmission of mechanical force-signals over a multicellular sheet and how force-induced re-organization of the actin cytoskeleton can result in morphological changes.

External mechanical forces can affect actin cytoskeletal organization and further cell shape, through cadherin-based cell-cell contacts or focal adhesions. In the papers of this thesis we specifically focus on AmotL2, connecting adherens junctions to radial actin filaments. By linking the actin cytoskeletons of adjacent cells, we hypothesize AmotL2 to enable the detection of- and response to mechanical forces from the environment. Mechanical forces are in various ways involved in the regulation of most developmental processes and can in addition affect the development of many diseases. Hence, revealing the mechanisms behind force detection, -transmission and the cellular processes it translates in to, can strongly help the understanding- and further the prevention and treatment of these diseases.

AmotL2 in prevention of atherosclerotic plaque formation

Atherosclerotic plaque formation is induced by flow disturbances causing alterations in the shear stress exerted onto endothelial cells of the vessel wall. Shear stress has previously been shown to induce actomyosin dependent endothelial cell shape changes (Tzima, Irani-Tehrani et al. 2005). Since we identified AmotL2 as a protein in control of actin cytoskeletal structure and endothelial cell shape, it would be of great interest to investigate the relation between shear stress and AmotL2 expression- and activity levels. Further, it would be interesting to explore whether AmotL2 controls endothelial cell shape, also in the adult aorta. If so, would then low AmotL2 expression levels predispose vessels to plaque formation? Furthermore, could plaque formation be prevented if AmotL2 levels were to be restored?

AmotL2 as a tumor target

When it comes to cancer research, most studies have focused on the force exerted on tumor cells from the underlying matrix. For example, enhanced ECM rigidity due to extensive collagen deposition has been correlated to tumor progression (Provenzano, Inman et al. 2008). Similarly, loss of cell-cell adhesion strength can promote tumor invasion (Perl, Wilgenbus et al. 1998). Loss of characteristic epithelial junctions (eg. E-cadherin) is commonly accompanied by gain of mesenchymal features (eg. N-cadherin). This cadherin switch has been related to phenotypical changes and invasion into the stroma/ vasculature (Yang, Wu et al. 2008). Interestingly the phenotypical switch appears to be associated to a concomitant change in force generation (Schneider, Baronsky et al. 2013). How the cadherin switch/ EMT affects the contractility of cells and their capacity to relay signals as a response to mechanical stimuli, still remains to be investigated. In Paper IV of this thesis we identify a shorter AmotL2 p60 isoform, specifically upregulated in hypoxic tissues. AmotL2 p60 expression causes loss of apical-basal polarity and sensitizes tumor cells to growth factor signaling, promoting an invasive phenotype. Perhaps also AmotL2 p60-mediated loss of polarity, affects the cells ability to produce force. I hypothesize, a better understanding of how cells are mechanically interlinked, to be important to develop new strategies for preventing/ -treating aggressive tumors.

Angiomotins and mechanosensory systems in tissue regeneration

Recently, the Angiomotins in conjunction with the transcriptional co-activator YAP, were shown to control cell differentiation in the mouse blastocyst. Subcellular localization and hence activity of YAP was previously shown to be regulated by mechanical forces. In addition, mechanical forces have been ascribed a role in control of progenitor cell differentiation, into osteoblast- or adipocyte fate respectively (Dupont, Morsut et al. 2011). This is of great interest, as bones along with skeletal muscles and tendons are organs constantly exposed to shear stress. Bone fractures are healed through regeneration, which is at least in part dependent on external mechanical forces. Accordingly high bone mass is correlated to increased physical activity, exposing the bones to mechanical forces (Huang and Ogawa 2010). Given the importance of force during physiological conditions in these organs, it is not surprising that lost ability to detect environmental forces can result in severe diseases, for example muscular dystrophy. Knowing how cells detect forces and further how they translate into modification of properties such as cell differentiation and –proliferation, would strongly help to in a controlled fashion regenerate damaged tissues and organs. This could be applied for example to prevent loss of bone mass and fractions in elderly people unable to perform physical activity, to regenerate muscles after muscle atrophy or cachexia and tendons commonly damaged in athletes.

Moreover, shear stress promotes differentiation of mesenchymal stem cells into vascular endothelial cells, a finding with potential implications for generation of vascular grafts (Park, Huang et al. 2007). Possibly in the future the same principle can be applied also to other cell types and organs. Hence, research on mechanosensory systems could contribute to the development of novel techniques for organ regeneration.

Heterogenous cell-cell interactions and force transmission

Our studies focus on homogenous interactions in between endothelial cells of the developing aorta and epithelial cells of the epidermis, respectively. I hypothesize similar interactions to take place also in between cells of different origin. As mentioned in the introduction, an organ consists of a diverse mixture of cells, all coordinating their

actions to support organ function. It would hence make sense also for cells of different type, to interact and connect through intercellular cytoskeletal linkage. For example endothelial cells and vascular smooth muscle cells, epithelial cells and smooth muscle cells of the airways, endothelial cells and macrophages during angiogenic sprouting.

During physiological conditions, the vascular tone is regulated through alternating contractions and relaxations of vascular smooth muscle cells or pericytes. Loss of the dynamic contractile behavior can result in hypertension. Also epithelial cells of the airways are associated to smooth muscle cells, which under physiological conditions are much more quiescent compared to their vascular counterparts. Instead their activation (contraction) can result in devastating effects, for example preventing passage of air during asthma. Perhaps defective force sensing in between two cell types can be one underlying factor causing loss of tissue dynamics resulting in hypertension and asthma respectively.

Macrophages have been ascribed roles both during anastomosis of angiogenic sprouts and further vessel regression. The macrophages have been shown to support the initial contact formation in between tip-cell filopodia. Perhaps their action is mediated through generation of forces, pulling on the actin cytoskeletal network of the filopodia. This idea is certainly interesting, as it would allow control of angiogenic sprouting- and vessel regression for example during tumor progression.

In conclusion, intercellular connections allowing transmission of forces over a multicellular structure are needed for assembly and unity of individual cells into a functional organ. Loss of the linker-proteins bridging the cells, results in loss of tissue structure and invasion of tumor cells into surrounding tissues. Understanding the mechanisms behind how the cells and further their contractile cytoskeletons are connected, could be of importance for both prevention and treatment of diseases caused by loss of mechanosensory structures/ protein complexes. By re-establishing functional force-detecting systems, hopefully damaged organs can be regenerated and aggressive tumors reverted.

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